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Evaluation of bacillus strains for plant growth-promotion potentials on corn *(Zea mays)*, wheat *(Triticum aestivum)*, and soybean *(Glycine max)*.

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EVALUATION OF BACILLUS STRAINS FOR PLANT GROWTH PROMOTION POTENTIALS ON CORN (*Zea mays*), WHEAT (*Triticum aestivum*), AND SOYBEAN (*Glycine max).*

by

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EVALUATION OF BACILLUS STRAINS FOR PLANT GROWTH PROMOTION POTENTIALS ON CORN (*Zea mays*), WHEAT (*Triticum aestivum*), AND SOYBEAN (*Glycine max).*

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University of Nebraska, 2018

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Plant growth-promoting rhizobacteria (PGPR) can increase plant growth and yield by facilitating nutrient availability, hormone production, and inhibiting plant deleterious microorganisms. Twelve strains of bacillus (endospore-forming bacteria belonging to the genera *Bacillus, Paenibacillus* and *Lysinibacillus)* isolated from wheat rhizospheres were assessed for plant-growth promotion attributes in greenhouse and laboratory experiments. The objectives were to assess each strain's potential to promote growth in corn, wheat, and soybean; and to determine whether the physiological traits expressed *in vitro* by the strains related to their effectiveness in promoting plant growth. Greenhouse experiments to assess growth-promotion potential were conducted by applying the strains to seed of the test crops and growing the plants in a nonsterile potting mix soil for one month. Eleven of the twelve strains increased corn growth significantly compared to controls, and four of the most efficacious strains on corn- *Bacillus megaterium* R181, *B. safensis* R173, *B. simplex* R180, and *Paenibacillus graminis* R200 - also increased the growth of soybean and wheat. These strains

caused higher growth stimulation on corn than on soybean and wheat. Shoot weights were frequently increased over 200% on corn compared to the controls, whereas shoot weight stimulation by these strains on soybean and wheat did not exceed 50%. The strains were also tested *in vitro* for traits associated with plant growth-promotion, including antagonism against bacteria and fungi, mineral nutrient conversion, and growth hormone production. None of the strains exhibited strong antagonism against fungi *in vitro* and few strains inhibited other bacteria. Most strains expressed indole acetic acid production and phosphate solubilization, suggesting that these mechanisms are more prevalent. No set of traits, however, was a predictor of high growth promotion efficacy. The expression of numerous traits *in vitro* also was not predictive of high plant growth-promotion activity. Some strains that expressed multiple traits *in vitro* exhibited low growth-promotion efficacy in pot tests, whereas one strain - R200 - that tested positive for only one *in vitro* trait showed high efficacy. This study showed that bacillus possess high potentials to increase plant growth, but their efficacy *in vivo* cannot be predicted by *in vitro* assays.

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CHAPTER I

LITERATURE REVIEW

1.0 Introduction

The need to produce more food has necessitated the intensive use of chemical fertilizers and pesticides in agriculture. This has led to pollution of surface and groundwater via leaching and run off through erosion. Consequently, there are public concerns arising from the overuse of agrochemicals. For example, if food crops containing residues of chemical pesticides are consumed, it may be hazardous to human health. So, substantial research efforts are now focused on finding new alternatives to supplement the use of chemicals in agriculture. An aspect of these efforts is to use beneficial soil bacteria to increase plant growth and productivity. Beneficial rhizobacteria which can be used to promote plant growth and yield are called plant growth-promoting rhizobacteria (PGPR). When PGPR are applied onto seeds or roots of plants, they may colonize the entire root system, utilize amino acids and sugars found in root exudates as source of nutrient and energy to initiate plant growth-promotion activities to increase plant growth and yield (Kloepper and Schroth, 1978). Inoculation of plant with PGPR can increase growth up to 500% (Kloepper et al., 1980) and yield up to 57% on different crops (Asghar et al., 2004; Khalid et al., 1997).

Generally, PGPR can increase plant growth directly by providing nutrients and plant growth hormones in the rhizosphere or indirectly by reducing the effects of plant pathogens (Ahemad and Kibret, 2014). Those soil bacteria that can suppress plant pathogens are often used as biocontrol agents (BCA) to control

plant diseases. Conversely, those biocontrol bacteria that also increase plant growth can be regarded as PGPR. But since not all BCA increase plant growth, the term PGPR is not applicable for describing all BCA. In this literature review, the term PGPR is used to describe any beneficial bacteria that can increase plant growth by direct or indirect mechanisms.

Many beneficial bacteria have been identified and developed into commercially available products for promoting plant growth (Crow, 2014; Junaid et al., 2013). There are also many ongoing studies focusing on evaluating new bacterial strains or improving the existing ones for effective plant growth promotion performance in the field. The bacterial genera most commonly researched and reported as PGPR include *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas,* and *Serratia* (Bhattacharyya and Jha, 2012). Current research is largely focused on endospore-forming, Gram-positive bacteria in the genera *Bacillus, Paenibacillus* and *Lysinibacillus.* These genera were formerly classified as *Bacillus* but later separated into different genera (Xu and Côte, 2003). This group of bacteria will be referred to as "bacillus" in this document. The stress-tolerant endospore provides bioformulations of bacillus with long shelf life and higher chances of survival under harsh storage and environmental conditions (Thomas, 2012; Mandic-Mulec and Prosser, 2011: Adesemoye et al., 2017). These advantages make them attractive options as PGPR products.

Many reviews and research studies have been published on the various taxonomic groups of PGPR (Adesemoye et al., 2017; Bhattacharyya and Jha, 2012; Gray and Smith, 2005; Lugtenberg and Kamilova, 2009; Kloepper, 1994). Because strains of bacillus are the subjects of research for this thesis, much of the focus in this literature review is on bacillus PGPR. Topics reviewed includes historical development of the PGPR concept, PGPR-plant associations, mechanisms of action, isolation and evaluation of bacterial strains for plant growth promotion, and limitations to the use of PGPR.

1.1 Historical development of the PGPR concept

The concept of using soil bacteria to enhance plant growth dates to 372–287 BC, when Theophrastus first suggested the use of soil mixtures to remediate soil defects. Later in 1888, Hellriegel and Wilfarth demonstrated that rhizobia in root nodules of legumes can convert atmospheric nitrogen into ammonia for use by plants (Bhattacharyya and Jha, 2012; McNear, 2013). Afterwards, between 1895 and 1909, Russian researchers initiated the term “bacterialization”, which means the treatment of seeds with different cultures of beneficial bacteria to improve plant growth. Their work led to the industrial production and general use of different cultures of *Bacillus* species and *Azoctobacter chroococum* to improve plant growth beginning from 1962 (Kloepper, 1994). In 1978, Schroth and associates in the United States, used the term PGPR to describe specific strains of bacteria that increased the yield of root crops after colonizing the root systems in

greenhouse and field experiments (Burr et al., 1978; Kloepper and Schroth, 1978).

1.2 PGPR-plant associations

The most common PGPR are those in the genera *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas,* and *Serratia* (Bhattacharyya and Jha, 2012). Some scientists classify these PGPR into two major groups based on their spatial relationship with plants. They grouped PGPR as either ePGPR or iPGPR. The term ePGPR means extracellular plant growth promoting rhizobacteria. These rhizobacteria live and function outside of plant root cells; in soil closely associated with roots (i.e. rhizosphere); on root surfaces (i.e. rhizoplane); or in spaces between cells of the root cortex (Gray and Smith, 2005). They are free living, feeding on amino acids and sugars found in root exudates as their source of energy and nutrients (Walker et al., 2003), and increase plant growth via direct or indirect mechanisms. Bacillus PGPR are classified in this group. Other examples include bacteria in the genera, *Pseudomonas, Erwinia, Caulobacter, Serratia, Arthrobacter, Micrococcus, Flavobacterium, Chromobacterum, Agrobacterium, Hyphomycrobium* (Gray and Smith, 2005). The term iPGPR means intracellular plant growth-promoting rhizobacteria. The term is applied to PGPR that live inside plant root cells as endophytes/symbionts. The majority of iPGPR are Gram-negative, rod-shaped, nodule-forming rhizobia (i.e. *Rhizobium, Bradyrhizobium, Sinorhizobium,*

Azorhizobium, *Mesorhizobium* and *Allorhizobium*), while a few exist as Gram-positive rod, cocci, or pleomorphic forms (Bhattacharyya and Jha, 2012; Gray and Smith, 2005).

1.3 Mechanisms of plant growth promotion

Generally, PGPR can increase plant growth via direct or indirect mechanisms. In both mechanisms, PGPR or their secondary metabolites alter the biotic and abiotic components of the rhizosphere community to bring about plant growth promotion (Gray and Smith, 2005; Lugtenberg and Kamilova, 2009; Kloepper and Schroth, 1978).

1.3.1 Direct mechanisms of plant growth promotion

Direct plant growth promotion is most evident when PGPR increase plant growth directly by providing growth factors and nutrients to plants (Vesey, 2003). The direct mechanism does not involve suppression of plant pathogens (Lugtenberg and Kamilova, 2009); it may involve biological processes such as biological nitrogen-fixation, solubilization of complex organic or inorganic nutrients, mobilization of iron via siderophore production, and production of plant growth regulators such as indole acetic acid (IAA), gibberellin and cytokinin. Based on different mode of actions, direct plant growth-promoting rhizobacteria can be grouped into three categories including biofertilizers, phytostimulators, and rhizoremediators (Lugtenberg and Kamilova, 2009).

1.3.2 Biofertilizers

There is no universally accepted definition for biofertilizers, but bacteria that can increase plant growth by supplying nutrients to plants were described as biofertilizers by Lugtenberg and Kamilova (2009). The specific activities of such bacteria may include nitrogen-fixation and/or solubilization of organic and inorganic nutrients, particularly phosphate and ferric compounds in the rhizosphere.

Nitrogen-fixation

Nitrogen is the most important soil nutrient required by plants. However, nitrogen is frequently lost from agricultural soil as nitrate via leaching, nitrogen gas via denitrification and volatilization, and in various other forms through crop removal and soil erosion (Lamb et al., 2014). Some PGPR can supply plants with nitrogen via biological nitrogen-fixation whereby atmospheric nitrogen is converted to ammonia in the soil using a complex enzyme system known as nitrogenase (Kim and Rees, 1994). Nitrogen-fixation may occur during symbiotic (rhizobia and *Frankia*) and non-symbiotic (free living, associative and endophytes) interactions between plants and PGPR (Ahemad and Kibret, 2014). Among non-symbiotic or free-living nitrogen-fixing bacteria that have been reported as PGPR are those in the genera *Azospirillum, Azotobacter, Bacillus, Gluconoacetobacter diazotrophicus* and *Azocarcus* (Ahemad and Kibret, 2014; Kim and Rees, 1994). Many studies have been reported for bacillus PGPR promoting plant growth via nitrogen-fixation. *Paenibacillus polymyxa* strain P2b-

2R was observed to increase both foliar N content and biomass of lodgepole pine seedlings in growth chamber experiment. The foliar N content and length of seedlings inoculated with the strain was up to 38% and 18% higher than the control seedlings, respectively (Tang et al., 2017). In another study, the same strain also increased the foliar-N content and biomass of canola (up to 118% and 90%, respectively) and tomato (up to 22% and 17%, respectively) plants significantly compared to control (Padda et al., 2016).

Phosphate solubilization

Most of soil phosphorus (P) is present in insoluble forms such as inorganic mineral form (apatite), or as one of several organic forms such as inositol phosphate, phosphomonoesters, and phosphotriesters (Glick, 2012). Plants only absorb P in two different soluble forms, as monobasic ($H_2P0^-_4$) or dibasic ($HP0^{2-}_4$) ions. These forms of P are generally present in a low amount in soil (Bhattacharyya and Jha, 2012). The low levels of soluble P in soil can limit the growth of plants. Some PGPR known as phosphate solubilizing bacteria, including many bacilli, can convert insoluble phosphate to soluble forms (Lugtenberg and Kamilova, 2009). These bacteria produce enzymes such as phosphatases, phytases, and organic acids to solubilize phosphorus from different sources such as rock phosphate (Rodriguez et al., 2006). For example, *Bacillus megaterium var. phosphaticum*, a phosphate solubilizing bacterium, increased plant growth, photosynthesis rate, and P availability in soil compared to controls in greenhouse experiments (Han and Lee, 2006). The strain increased growth up

to 22% and 27% and up to 26% and 29%, respectively for cucumber and pepper shoot growth and root dry weight. In field experiments, the same strain also increased the dry mater of lettuce and made more P available in soil when compared with controls. Also, in a growth chamber experiment using P-deficient soil amended with rock phosphate, many phosphate-solubilizing bacilli increased significantly the numbers of pods, pod weight, plant height, and seed yields of treated canola plants compared to the controls (De Freitas et al., 1997). The study showed that *B. thuringiensis* strain 2P1M3 significantly increased seed yield by 35%, pod weight by 25%, and number of pods by 30–54% in treated plants compared to the controls on P-deficient soil without rock phosphate.

Siderophore production

Iron is an essential nutrient for plants. In aerobic environments, iron occurs in ferric iron (Fe^{3+}) form, a form that has a high tendency to form insoluble hydroxides and oxyhydroxides. This often makes iron inaccessible to both plants and some microorganisms (Rajkumar et al., 2010). However, some bacteria including bacilli have mechanisms through which they can acquire the inaccessible iron. This mechanism involves the secretion of different forms of low-molecular mass iron chelators known as siderophores. Siderophores have high affinity for binding with ferric iron (Fe^{3+}). After binding with siderophore, ferric iron (Fe^{3+}) is reduced to ferrous (Fe^{2+}) iron in the bacteria cell membrane. Within the rhizosphere, plants can absorb iron from soil and microbes via different mechanisms such as chelation, through ligand exchange reaction or by

direct uptake of ferric iron-siderophore complexes (Ahemad and Kibret, 2014). Generally, it is believed that siderophore-producing bacteria support plant growth via siderophore-mediated competition against deleterious microbes in the rhizosphere (Compant et al., 2005). The involvement of siderophore-producing bacilli for the growth promotion of plants has been documented. *Bacillus pumilus* 8N-4 was found to exhibit several plant growth-promoting traits including siderophore production. Inoculation of wheat with the strain resulted in significant increases in plant biomass, root length and many other growth parameters (Hafeez et al., 2006). The study, however, did not show that the growth promotion by the bacillus strain was due to the direct effect of siderophores. However, direct plant growth promotion resulting from siderophore-producing pseudomonas has been reported. In a greenhouse study conducted using an iron-deficient calcareous soil, maize (corn) seeds were bacterized with siderophore-producing *Pseudomonas* species, strains GRP3A and PRS9, with the goal of developing a system suitable for iron acquisition under iron-stressed conditions. It was observed that the strains increased both germination and growth of the treated seeds significantly compared to controls (Sharma and Johri, 2003). This supports the possible role of bacterial siderophores in direct plant growth promotion.

1.3.3 Phytostimulators

Any strain of PGPR is considered a phytostimulator if it increases plant growth by producing plant growth regulators such as indole acetic acid (IAA), gibberellic acids, and cytokinin in the rhizosphere (García-Fraile et al., 2015).

Indole acetic acid (IAA), gibberellin and cytokinin

Some PGPR can produce plant growth hormones such as IAA (Kravchenko et al., 2004), gibberellin (Joo et al., 2005), and cytokinin (Kaymak, 2010) in the rhizosphere. In the presence of a considerable amount of tryptophan, the precursor to IAA, some PGPR can produce IAA, an indispensable plant growth hormone (Kravchenko et al., 2004; Teale et al., 200). Indole acetic acid producing-PGPR, *B. amyloliqufaciens* FZB42, was shown to have the ability to promote the growth of duck weed in the presence of tryptophan in a microtiter plate assay (Idris et al., 2007). A mutant strain that produced lesser IAA than the wild type strain was less efficient in promoting plant growth than the wild type, and an IAA-deficient mutant did not increase growth (Idris et al., 2007). It was also found that the amount of tryptophan supplied can affect plant growth promotion by the IAA-producing PGPR.

Gibberellins are important plant hormones involved in many developmental and physiological processes in plants. The ability of gibberellin-producing bacteria to promote plant growth has been documented. For example, the growth of red pepper plants was significantly enhanced by gibberellin-

producing bacterial strains *B. cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ- 69 (Joo et al., 2005).

Cytokinins are a class of plant growth hormones produced by plants and some microorganisms. Cytokinin plays an essential role in regulating cytokinesis, growth and development in plants (Aloni et al., 2006). The supply of cytokinin in the rhizosphere by plant-associated bacteria can result in increased plant growth (Ortíz-Castro et al., 2008; Aloni et al., 2006). Plant growth promotion induced by cytokinin-producing bacteria has been well documented for several rhizobacteria species including bacillus. The cytokinin-producing bacterium, *B. megaterium* (UMCV1) was shown to promote the growth of *Arabidopsis thaliana* and *Phaseolus vulgaris* plants *in vitro* and in soil (Ortíz-Castro et al., 2008). The strain increased lateral root number, lateral root growth and root hair length of the inoculated plants compared to control. Arabidopsis mutants lacking putative cytokinin receptors were insensitive to the growth promotion effect exerted by the strain, further demonstrating that cytokinin was responsible for the growth promotion observed.

Activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme

The mode of action of some PGPR involves the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that could cleave ACC, the immediate precursor to ethylene synthesis in plants. Ethylene production in plants can slow down root growth in stressed environments. Production of the enzyme ACC deaminase by some PGPR could reverse this by

decreasing ethylene production in the roots of host plants, resulting in root elongation and enhanced plant growth. Thus, PGPR boost plant growth, particularly under stressed conditions by the regulation of accelerated ethylene production in response to abiotic and biotic stresses such as salinity, drought, waterlogging, temperature, pathogenicity, and contaminants (Saleem et al., 2007; Kaymak, 2010). For example, Zahir et al. (2009) identified three ACC deaminase-producing PGPR strains including *P. putida* (N21), *P. aeruginosa* (N39), and *Serratia proteamaculans* (M35) that induced a significant root growth of inoculated plant under salinity stress. It was suggested that the 1-aminocyclopropane-1-carboxylic acid-deaminase activity of the strains might have caused a reduction in the synthesis of stress (salt)-induced inhibitory levels of ethylene.

1.3.4 Rhizoremediators

Apart from increasing plant growth to increase yields, some PGPR are used to stimulate plant growth for soil remediation. The sets of PGPR that are used for increasing plant growth for environmental rhizoremediation are called Rhizoremediators (Kuiper et al., 2001). They can use root exudates of plants grown in polluted soils as their source of nutrients and energy to degrade the soil pollutants. Rhizoremediators are particularly useful in phytoremediation strategies to extract, immobilize, contain and/or degrade soil contaminants (Gerhardt et al. 2017). According to the study by Kuiper et al. (2001), inoculation with naphthalene-degrading bacterium, *Pseudomonas putida* PCL1444 effectively

protected plant against naphthalene toxicity, whereas un-inoculated plants died from naphthalene toxicity.

1.3.5 Indirect mechanisms of plant growth promotion

According to Zablotowicz et al. (1991), indirect mechanisms of growth promotion involves the reduction of population densities of deleterious microbes including major and minor pathogens, and other deleterious organisms. Essentially, biocontrol of pathogens and deleterious rhizospheric microbes reverses yield loss caused the deleterious organisms (Yuen and Schroth, 1986). Indirect growth promotion may occur via direct antagonism such as antibiotic and lytic enzyme activity, or via competition for nutrients, niche exclusion and induced systemic resistance in host plants (Bhattacharyya and Jha, 2012; Glick, 2012; Adesemoye and Egamberdieva, 2013; Zandi and Basu, 2016). Many researchers have conducted studies on different indirect plant growth promotion mechanisms. As reviewed here, some studies showed that indirect mechanisms only exhibited biocontrol effects on plants without increasing plant growth but there are other cases where disease control by biocontrol PGPR resulted in plant growth promotion.

1.3.6 Antibiotics and lytic enzymes production

Antibiotics consist of heterogeneous groups of low-molecular-weight secondary metabolites that are deleterious to the growth or metabolic activities of other microbes including plant pathogens (Beneduzi et al., 2012). Diverse

antibiotics are produced by bacteria including bacilli-PGPR to antagonize many phytopathogens (Glick et al., 2007; Beneduzi et al., 2012). These antibiotics may include compounds such as amphisin, 2, 4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, and tropolone. They can be grouped as volatile (e.g. hydrogen cyanide) or diffusible antibiotics such as phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides (Beneduzi et al., 2012). They kill pathogens by interfering with the integrity of the cell wall, cell membrane, and cytoplasm of the pathogen cells. Their specific activity on the pathogen cells may include cell wall synthesis inhibition, influencing cell membrane structures, or inhibition of ribosomal complex formation in the pathogen cells (Maksimov et al., 2011). Production of one or more of these antibiotics by PGPR in the rhizosphere can suppress pathogen effects and reverse disease caused by deleterious microbes. In many cases, disease suppression may not result in plant growth promotion but in some cases, it does bring about a significant increase in plant growth. For example, in detached leaf and seedling assays, four *Bacillus subtilis*, strains UMAF6614, UMAF6616, UMAF6639, and UMAF8561, producing iturin and fengycin, were found to be suppressive to powdery mildew of cucurbits caused by *Podosphaera fusca* on melon (Romero et al., 2007). To further support that antibiosis was the major factor in the disease suppression exhibited by the strains, three lipopeptide antibiotics including surfactin, fengycin, and iturin A or bacillomycin were identified in butanolic extracts from cell-free culture filtrates of the strains. The disease suppressions by these strains were not shown to cause plant growth

increase *in vivo*. However, in greenhouse and field studies conducted by Kloepper and Schroth (1981), five strains of *Pseudomonas* species exhibiting antibiosis *in vitro*, caused significant increases ranging from 300 to 500% in total weight of potato plants grown in nonsterile field soils. Mutants without antibiosis did not increase plant growth. Furthermore, wild-type strains resulted in reductions in root zone fungal and Gram-positive bacteria population densities ranging from 23% to 64% and 25% to 93%, respectively. But no differences were detected in microbial populations on roots of plants treated with mutants having no antibiosis activity.

Similarly, enzymes produced by some bacteria including biocontrol bacillus PGPR are implicated in indirect plant growth promotion. Microbial enzymes such as chitinases, cellulases, β-1, 3 glucanases, proteases, and lipases can lyse a portion of cell walls of many plant pathogenic fungi (Glick, 2012). Biocontrol agents that synthesize one or more of these enzymes have been found to exhibit disease suppressive activity against a range of pathogenic fungi; supporting plant growth or leading to plant growth promotion. For example, antifungal and chitinolytic *Bacillus circulans* GRS 243 and another biocontrol bacterium, *Serratia marcescens* GPS 5, were antagonistic against *Phaeoisariopsis personata* during *in vitro* tests. The strains were shown to suppress the late leaf spot (LLS) disease caused by *P. personata* on peanut both in greenhouse and field studies. Furthermore, in the same study, purified chitinase of strain *S. marcescens* GPS 5 inhibited the *in vitro* germination of *P. personata* conidia, lysed the conidia, and effectively controlled LLS in greenhouse tests (Kishore et al., 2005).

Also, an effective biocontrol mixture comprising of three chitinolytic bacteria including, *Serratia plymuthica* C-1, which was strongly antagonistic to *Phytophthora capsici*; *Chromobacterium sp*. C-61, which was strongly antagonistic to *Rhizoctonia solani*; and *Lysobacter enzymogenes* C-3, which was antagonistic to *R. solani* and *Fusarium* spp were shown to effectively suppressed *Phytophthora* blight of pepper in greenhouse pot and fields experiments (Kim et al., 2008). The bioformulations used in the study were developed from the bacterial cultures grown from a chitin medium. These studies show that enzymes produced by biocontrol PGPR were involved in plant disease suppression to support plant growth and health as a biocontrol agent, but there was no documentation of any plant growth promotion activity by the strains.

1.3.7 Competition for nutrients and niche exclusion

Competition for nutrients and niche exclusion is another mechanism involved in indirect plant growth promotion. PGPR acting through this mechanism express fast chemotactic movement along growing root or produce substance such as siderophores that enables them to rapidly use nutrients and growth factors such as iron found in root exudates more quickly than other organisms present in the root zone. These thereby cause the PGPR to outcompete the pathogens, excluding them from available nutrients and niches on the root (Lugtenberg and Kamilova, 2009). This activity may not result in increased plant growth but support plant growth by reducing population densities of deleterious microbes around plant roots. For example, treatments of carnation roots with

bacterial strain *Pseudomonas* spp. WCS417r significantly reduced fusarium wilt disease caused by *Fusarium oxysporum f. sp. dianthi.* A mutant strain defective in siderophore biosynthesis was comparatively less effective in disease suppression. Hence, the disease suppression exhibited by the wild-type strain was due to competition for iron between the biocontrol strain and the pathogen (Duijff et al., 1993). Furthermore, *Collimonas fungivorans,* a Gram negative, rod shaped bacterium was observed to suppress tomato foot and root rot (TFRR) disease caused by *Fusarium oxysporum* under greenhouse conditions in potting soil. The visual observation of the fluorescently labelled strain on the plant root showed that the bacterial strain occupied the same sites on the root as did TFRR. It was assumed that *C. fungivorans* mainly controls TFRR through a mechanism of competition for nutrients and niches (Kamilova et al., 2007). In the above examples, competition for nutrient did not improve plant growth. However, in a study involving siderophore-producing *Bacillus subtilis* strain CAS15, the strain reduced Fusarium wilt incidence and increased growth of pepper in pot culture experiments. Disease suppression and growth promotion were due to competition for iron nutrient between the strain and the pathogen in the rhizosphere. Growth increase was up to 55% for plant height, 37% for fruit weight and 50% for average yield per plant. It was noted by the authors that when study was conducted in soil supplemented with iron, disease suppression by the strain was reduced (Yu et al., 2011).

1.3.8 Induced systemic resistance

Induced systemic resistance (ISR) is another indirect mechanism of plant growth promotion through suppression of diseases caused by pathogens (Duijff et al., 1993). In ISR, PGPR stimulate the host plant's defenses, thereby reducing the level of disease from infection by pathogens with the defense occurring throughout the plant (Kloepper, 1996). The PGPR triggers immune defense in plant roots that spread systemically throughout the plant and enhance the defensive capacity of other parts of the plant against subsequent infection by the pathogens (Van Loon and Bakker, 2005). ISR is mediated by jasmonate (JA) and ethylene (ET)-sensitive pathways (Walters et al. 2013). ISR is different from systemic acquired resistance (SAR) which can be induced by treatment with a pathogenic microbe and mediated by a salicylic acid (SA)-dependent process. Generally, ISR confers protection against a broad spectrum of plant pathogens (Kilic-Ekici and Yuen, 2003; Van Loon and Bakker, 2005). Studies involving elicitation of ISR by PGPR are commonly reported for *Pseudomonas* spp. and other gram-negative bacteria (Van Peer et al., 1991; Raupach et al., 1996). A comprehensive review of studies on induced systemic resistance and promotion of plant growth by *Bacillus* spp. has been published (Kloepper et al., 2004). It reviews ISR elicitation by a long list of *Bacillus* species including *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus.* In one study, inoculation of roots of grapevine with living cells or extracts from *B. substillis* strain Bs-271 elicited a weakly ISR against *Botrytis cinerea* on grapevine leaves. In another study conducted by Krause et al. (2003),

eleven bacterial strains were isolated from compost, and screened for ISR against *Xanthomonas campestris* pv. a*rmoraciae* bacterial leaf spot on radish. All the bacterial strains elicited significant protection against the pathogen. Four *Bacillus* spp. were among the top performing strains. Another four bacterial strains; *B. pumilus* SE34, *B. pumilus* T4, *P. fluorescens* 89B61, and *S. marcescen* 90–166 applied separately into potting mix, significantly suppressed bacterial leaf spot caused by *P. syringae pv. maculicola* on *Arabidopsis thaliana* (Ryu et al., 2003). Also, live or heat-killed cells of *Lysobacter enzymogenes* C3, a Gram negative, rod-shaped biocontrol strain, when applied to tall fescue and wheat roots, elicited a long lasting, ISR expressed in the foliage against fungal *Bipolaris sorokiniana* of tall fescue and *Rhizoctonia solani* in wheat (Kilic-Ekici and Yuen, 2003). In these examples, it was not shown that elicitation of ISR caused plant growth promotion to occur on the treated plants. It appears that, in most studies, the effects of induced systemic resistance on plant growth increase are not evaluated

1.4. Isolation and evaluation of bacterial strains for plant growth promotion

1.4.1 Requirements for effective PGPR activity

In searching for effective PGPR strains, there is a need to consider the rhizosphere competence ability of the strains. Rhizosphere competence is the ability of bacteria to aggressively colonize and flourish in the rhizosphere with high survivability (Zablotowicz et al., 1991; Adesemoye and Egamberdieva, 2013). Kloepper et al. (1980) have described the effects of rhizosphere colonization by PGPR strains on potato plants in field studies. They found that

mutant strains of the PGPR resistant to antibiotics colonized the entire rhizosphere of treated potato plants, including the developing daughter tubers and apical roots of adjacent nontreated plants. The PGPR populations in the rhizosphere were as great as 9.6×10^5 colony forming units per centimeter (cfu/cm) of root up to 2 weeks after plant emergence and averaged 10^3 cfu/cm throughout the growing season. The PGPR strains significantly increased potato growth up to 500% greater than controls.

1.4.2 Sample collection for isolating potential PGPR

The method used for the collection of samples for isolating potential PGPR strains is important in determine the effectiveness of the PGPR strains. Some of the questions that need to be answered prior to collecting samples for PGPR isolation include; on what plant will the PGPR strain be applied? What would be the purpose of the PGPR; would it be used primarily as a biofertilizer, phytostimulator, or would it be applied primarily in nutrient rich agricultural fields? Is it intended to be used in greenhouse production or in fields? What are the prevailing environmental conditions in the locations in which the PGPR would be used? The answers to these questions will determine where and when to collect the samples for PGPR isolation.

On what plant will the PGPR strain be applied?

Strains of PGPR that will be effective in increasing plant growth must be able to colonize plant roots (Kloepper et al., 1980). Root colonization by PGPR

strains can be host specific, crop specific, or cultivar specific (Kloepper 1996). In other words, strains that aggressively colonize one tomato hybrid may not be good colonizers of a different tomato hybrid. For example, in a study involving inoculation of *P. aeruginosa* PNA1 unto pigeonpea and chickpea plants of two genotypes; susceptible and moderately tolerant to fusarium wilt. It was shown that the strain significantly reduced the disease up to maturity in moderately tolerant genotypes, but the susceptible genotypes were not protected up to maturity. The colonization of the plant roots by PNA1 was measured using a *lacZ*-marked strain of the bacterium. It was observed that root colonization was ten-fold lower on the susceptible genotypes than on the moderately tolerant genotypes, indicating that differences in plant genotypes may affect bacteria root colonization (Anjaiah et al., 2003). It is therefore essential to consider the plant host upon which the potential PGPR will be applied. Thorough screening for plant growth promotion effects across different plant varieties or cultivars with different genotypes might be needed to identify very promising strains when prospecting for PGPR strains for commercialization purposes.

What is the specific purpose and environmental conditions of the location of use of the PGPR?

It is generally thought that there is a higher chance of finding PGPR strains that will be effective for indirect plant growth promotion through pathogen suppressions or control of deleterious microorganisms from disease-suppressive soils. Weller (1998) showed that the percentage of fluorescent pseudomonads

suppressive to take-all disease in greenhouse bioassays was greater when the bacteria were isolated from roots of wheat grown in fields suppressive to take-all. Similarly, strains isolated from a field with specific environmental conditions such as extreme temperature, pH, moisture content, soil organic matter content, high salinity, or soil contaminants might exhibit more effectiveness when utilized under similar conditions. In other words, strains isolated from nutrient deficient soil may have higher potentials for direct plant growth promotion through better nutrient uptake. For example, the plant growth-promoting-rhizobacterium, *Pseudomonas putida* GR12-2 isolated from the rhizosphere of plants growing in the Canadian High Arctic was reported to be able to grow and promote root elongation of both spring and winter canola at 5°C, a temperature at which only a relatively small number of bacteria can proliferate and function (Sun et al., 1995). *Pantoea dispersa* strain 1A, a Gram-negative rod-shaped bacterium isolated and able to grow at 4°C was reported to positively influence and promote the growth and nutrient uptake parameters of wheat growing in cold environments (Selvakumar et al., 2008). These indicate that the chance of selecting effective strains may be improved by isolating the strains from the same environment in which they will be used (Weller, 1988). Hence, consideration for the intended purpose and the environmental conditions in the location of use is critical to finding effective PGPR strains.

1.5.0 Concerns affecting general use of PGPR

There are some concerns limiting the general use and acceptance of PGPR. Inconsistent results of plant growth promotion in fields by PGPR is a concern. Several studies have demonstrated or reiterated the inconsistent performance of PGPR strains in fields (Adesemoye et al., 2017; Weller, 1988) as a major impediment to the general acceptance of PGPR agents. Also, there is a concern of incompatibility of PGPR with existing farming practices, particularly agrochemicals. The results of the pesticide-PGPR compatibility studies conducted by Zablotowicz et al. (1992) showed that bacteria strains were not always compatible with chemical seed treatments *in vivo*. There is also the limitation of narrow spectrum activity, need for special storage condition for PGPR formulations, and susceptibility of PGPR strains to several biotic and abiotic factors (Beneduzi et al., 2012; Weller, 1988).

1.6.0 Factors influencing PGPR activity

The effectiveness of a PGPR strain can be affected by several environmental biotic and abiotic factors. Host plant effects and competition with indigenous microbes are examples of biotic factors. Zhang et al. (2014) demonstrated the influence of host factor on PGPR activities. In the study, PGPR strain *B. amyloliquefaciens* SQR9, isolated from cucumber rhizosphere and *B. subtilis* N11, isolated from banana rhizosphere, were found to be more effective when applied to the plants from which they were originally isolated compared to when used on another plant. The variability in effectiveness was attributed to

variation in the adaptation of the PGPR strains to convert the root exudates of the plant host to plant growth promoting factors (Zhang et al., 2014). Abiotic factors that could affect PGPR activities include several environmental conditions, such as soil type, temperature, moisture content, soil organic matter, and pH (Cakmakçi et al., 2006; Banerjee et al., 2006; McSpadden-Gardener, 2004). Studies have demonstrated that a PGPR strain that perform well in one location failed to produce growth promotion effects in other locations owing to differences in environmental conditions. In their study in two field locations, Suslow et al. (1979) showed that one PGPR strain when inoculated onto sugar beet increased yield in one California field location but failed consistently when tested in Idaho. Another PGPR strain that caused great yield benefits in Idaho had no significant effect in multiple California trials. This work indicated that PGPR are more consistently effective when utilized as treatments in the same region or in regions having similar environmental conditions to where they were isolated (Weller et al., 1985).

1.7.0 Research objectives

This study is part of a larger University of Nebraska-Lincoln project to identify and develop PGPR for use in Nebraska's diverse cropping systems, with a focus on bacillus strains. In this context, 'Bacillus' refers to bacteria belonging to Gram positive, endospore-forming bacteria genera including *Bacillus*, *Paenibacillus*, and *Lysinibacillu*s species, because these group of bacteria have better physiological traits advantages that enable them to persist even under harsh

environmental conditions (McSpadden-Gardener, 2004; Kumar et al., 2011). Recently, a commercial seed treatment involving strains of *B. firmus* (I-1582) that was isolated from Israel soil was evaluated for control of soybean cyst nematode in several locations in Nebraska, but the product was ineffective against the nematode and had no effect on yield in any of the locations (Musil, 2016). This current work is the first study in which several bacillus PGPR strains isolated from Nebraska soil are evaluated extensively. The strains used in this study were isolated from the rhizosphere of wheat grown near North Platte, West Central Nebraska. There were two objectives in this study. One objective was to assess the plant growth-promotion potentials of the twelve bacterial strains in greenhouse experiments on sweetcorn, soybean, and wheat, the three crops most common in Nebraska. The second objective was to examine the relationship of *in vitro* physiological traits of the strains to their growth promotion efficacy.

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CHAPTER II

EVALUATION OF BACILLUS STRAINS FOR PLANT GROWTH PROMOTION POTENTIALS ON CORN (*Zea mays*), WHEAT (*Triticum aestivum*), AND SOYBEAN (*Glycine max).*

2.1 Introduction

There is increasing need to use plant growth promoting rhizobacteria (PGPR) to enhance crop production through the facilitation of nutrient availability and/or the suppression of plant pathogens. “Bacillus” is the taxonomic group that has been most widely studied as PGPR, and currently the most commonly commercialized as plant growth enhancers and biological control agents. In the context of this study, bacillus refers to as any rod-shaped, endospore-forming Gram-positive bacterium that was previously classified in the genus *Bacillus*. The genus was divided into several genera including *Bacillus, Paenibacillus* and *Lysinibacillus*. These groups of bacteria have in common the ability to produce dormant, heat, and desiccation-tolerant spores. This trait enables them to survive and persist under harsh conditions in the field. It gives commercialized bacillus-based biological products the potential for an extended shelf life (Schwartz et al., 2013). Other advantageous traits possessed by this group include multilayer cell wall structures that contribute to stress tolerance, the ability to secrete antibiotics, extracellular enzymes and other molecular signals (McSpadden-Gardener, 2004; Kumar et al., 2011), as well as the ability to live as facultative anaerobes and exist in many extreme environments (Silini-Cherif et al., 2012).

Many studies have described bacillus strains as effective PGPR agents (Gutiérrez- Mañero, et al., 2001; Zheng et al., 2013; Lugtenberg and Kamilova, 2009; Kumar et al., 2011; Kumar et al., 2012). Strains that are effective for plant growth-promotion and which are most frequently reported belong to *Bacillus subtilis, B. megaterium, B. licheniformis, B. amyloliquefaciens, B. pumilus and Paenibacillus polymyx*a etc. (Çakmakçı et al., 2007; Joseph et al., 2012). These strains have exhibited a variety of plant growth promotion effects on many crop species. For instance, *B. amyloliquefaciens* KPS46 increased root and shoot lengths and plant biomass of soybean plant compared to the control (Buensanteai et al., 2008). Similarly, *B. subtilis ALB629* stimulated both foliar and root growth of cacao when inoculated onto cacao seedlings (Falcäo et al., 2014). In another study, *B. megaterium* mj1212 increased shoot length, root length and fresh weight of mustard plants (Kang et al., 2014). In addition, *Paenibacillus polymyxa* P2b-2R, a nitrogen-fixing strain enhanced the growth of canola, an important oilseed crop (Puri et al., 2016).

The effectiveness of a PGPR strain can be affected by many biotic and abiotic environmental factors. Biotic factors that can affect PGPR growth and effectiveness may include host plant effects and competition with indigenous microbes (Zhang et al., 2014). Abiotic factors may include soil conditions such as soil type, temperature, moisture content, organic matter, and pH (Cakmakçi et al., 2006; Banerjee et al., 2006; McSpadden-Gardener, 2004). Many studies have demonstrated that a PGPR strain that performs well in one location might fail to produce significant growth promotion effects in other locations owing to

differences in environmental conditions between locations. In a study involving multiple locations, Suslow et al. (1979) found that one PGPR strain increased sugar beet yield in California field tests but failed consistently when tested in Idaho, whereas another strain that caused greatest yield benefits in Idaho had no significant effect in some California trials. Recently, a commercial seed treatment involving strains of *Bacillus firmus* (I-1582) that was isolated in Israel was evaluated for control of soybean cyst nematode in several Nebraska locations, but the product was ineffective against the nematode and had no effect on yield in any of the locations (Musil, 2016). Also, as an example of PGPR activity being affected by plant species, strain *B. amyloliquefaciens* SQR9, isolated from cucumber rhizosphere and *B. subtilis* N11, isolated from banana rhizosphere, were found to be more effective when applied to the plant species of origin as compared to the other plant species (Zhang et al., 2014).

This study is part of a larger University of Nebraska-Lincoln project to identify and develop PGPR for use in Nebraska's diverse cropping systems. The focus is being placed on developing bacillus strains because this group of bacteria have more physiological traits that enable them to persist under harsh environmental conditions (McSpadden-Gardener, 2004; Kumar et al., 2011). There had been no prior report of bacillus PGPR strains originating from Nebraska, nor any extensive evaluation in Nebraska of bacillus PGPR strains originating from other areas of the United States. In a study involving field evaluations of a commercial PGPR products in various locations in Nebraska, it was found that the PGPR product failed to increase a significant growth in all the

tests trials (Musil, 2016). Working with the presumption that the most effective strains for application in Nebraska would be found among those isolated from Nebraska (Weller et al., 1985), Dr. Tony Adesemoye isolated some bacillus strains from the rhizosphere of wheat grown in Nebraska. These strains are the subject of this study. There were two objectives in the research reported in this chapter. The first was to determine whether any of the bacillus strains has potential for enhancing plant growth using sweetcorn as a plant system. The second was to identify which of the strains would also be efficacious on soybean and wheat, the other crops common to Nebraska.

2.2 Materials and Methods

2.2.1 Strains and general bacteriological methods

Twelve bacterial strains were isolated from the rhizosphere of wheat plants grown in North Platte, NE by Dr. Tony Adesemoye, University of Nebraska-Lincoln. They were identified through 16s rDNA sequencing as *Bacillus acidiceler* R228 (Genbank Accession Number: KY515411); *B. megaterium* strains R181 (KY807994) and R232 (KY515414); *B. pumilus* strains R174 (KY515394), R183 (KY515399), and R190 (KY515404)*; B. safensis* strains R173 (KY5153930) and R176 (KY515395);*B. simplex* R180 (KY515398); *Lysinibacillus macrolides* R198 (KY515408); *Paenibacillus cineris* R177 (KY515396); and *P. graminis* R200 (KY515409).

Each strain was stored at -75°C in a storage broth containing (g/L) tryptone (10), yeast extract (5), NaCl (0.5), $K_2HPO_4 \cdot 3H_2O$ (6.3), KH_2PO_4 (1.8),

$Na_3C_6H_5O_7$ (0.45), $MgSO_4.7H_2O$ (0.09), $(NH_4)_2SO_4$ (0.9) and glycerol (64 mL). Cultures were prepared monthly from frozen storage by streaking each strain onto 10% tryptic soy agar (TSA) medium. Inoculum suspension for seed treatment was prepared by evenly spreading a single colony of a bacterial strain onto the surface of a 10%TSA plate and incubating the culture for 36 to 48 hours at 28°C The bacterial cells were washed off the plate with 5 mL sterile phosphate buffer (PB) using a sterile spatula into a sterile test tube. Following vortexing, a spectrophotometer was used to measure the absorbance (600 nm) of the cell suspension, which was then diluted to 10^8 cfu/mL with sterile PB.

2.2.2 Seed treatment and sowing

Seeds of sweetcorn (Sugar Buns f1 se+, Johnny's Selected Seeds), wheat (Overland W5-52, Huskers Genetics) and soybean (Vikings 2265, Johnny's Selected Seeds) were surface disinfected by soaking in 2% commercial bleach solution for 3 minutes and rinsed with sterile distilled water for at least five times (Gholami et al., 2009). Seeds were left to dry aseptically in a laminar air-flow hood and kept at 4°C for later use. Surface disinfected corn and wheat seeds were treated with bacterial strains by soaking in cell suspension for 60 minutes, while soybean seeds were soaked in cell suspensions for 30 minutes. Seeds were soaked in sterile PB as the no-bacteria control. Populations of bacterial cells adhering to the seeds after soaking were estimated by washing some treated seeds in sterile PB, and the liquid from the seed-wash used to conduct cell population assay using an 8-spot bacterial cell enumeration method (Yuen et al., 1991).

2.2.3 Greenhouse pot tests for growth promotion

Seeds were sown into a non-pasteurized potting-mix containing a mixture of loamy soil and sand at 2 to 1 ratio by volume. The results from a commercial analysis of the potting mix are provided in the Appendix. One corn seed was sown per pot, 3 soybean seeds were sown per pot and 5 wheat seeds were sown per pot. There were eight to five replicate pots for each seed treatment. Pots were arranged in a completely randomized design on a bench in a greenhouse where temperatures varied from 24°C (night) to 31°C (day). Each experiment lasted for 20 days during which pots were watered once a day without fertilization. At the end of the experiment, soil was carefully washed off the plant roots under running tap water and then the shoots and roots were separated. Shoot height, fresh and dry shoot weight, fresh and dry root weight were measured. Dry weights were determined after drying for 3 days at 70 °C.

2.2.4 Statistical analysis

Two data analysis procedures were applied to analyze the data from all growth promotion experiments using Statistical Analysis System (SAS; SAS institute, Cary NC) software. Dunnett's test was used to compare each bacterial treatment separately with no-bacteria control. Bacterial treatments were compared with each other by first conducting analysis of variance (ANOVA) to determine if there was a significant treatment effect, compared to the control. Then, mean

separation was performed using the LSD test ($\alpha \leq 0.05$) when a significant treatment effect was found in the ANOVA.

Percentage growth increase was determined for individual strains. It represents the amount of growth promotion (in percentage) that was induced by a strain for a growth variable compared to the control. It was calculated by using the equation $\frac{Mt-Mc}{Mc} X\ 100$, where Mt and Mc are the mean measurements of the treatment and control, respectively.

Likewise, the growth stimulation frequency (GSF) was calculated for each strain. It represents the rate, expressed as a percentage, at which a strain significantly increased the growth (at ≥ 95% confidence level) of a variable (e.g. shoot height, shoot weight and root weight) across all trials. The GSF was used to denote how consistently a strain increased significant growth across all trials. It was calculated for a strain by dividing the number of cases where a significant growth increase was induced by the strain by the total number of trials in which the strain was tested and then multiplied by 100.

Another set of calculations were "frequency in top 3" (FIT3) and "frequency in top 2" (FIT2). The FIT3 and FIT2 were the percentage of cases (growth measurements) in which a strain was among the three highest strains in the corn experiment and was among the two highest strains in the soybean and wheat experiments, respectively.

After preliminary analysis, significant treatment effects were found more consistently when using fresh biomass measurements compared to dry biomass measurements. Only fresh weight measurements were thus reported.

2.3 Results

2.3.1 Evaluation of strains for growth promotion on sweetcorn

All the 12 bacillus strains exhibited the potential to enhance sweetcorn growth compared to the control (Figure 2.1). Using Dunnett's test to compare individual strains with the control, each of the strains caused significant increase of one or more growth variable in at least two trials (Table 2.1). *B. simplex* strain R180 showed the highest growth stimulation frequency (GSF), followed by *B. safensis* R176 and *B. megaterium* strain R181, inducing GSF of 100, 83 and 78% respectively, of various growth variables across all trials. Other strains induced growth stimulation frequencies that varied from 33 to 67% of the growth variables across all trials.

Large variations were observed in the percentage growth increase by strains from trial to trial. For example, strains R181 and R180 increased shoot height growth that ranged from 18 to 45% and 30 to 41%, shoot weight growth from 40 to 140% and 68 to 118% while root weight growth increase ranged from 32 to 136% and 112 to 206% respectively. The highest growth promotion was observed on root growth compared to shoot growth. While the highest mean percentage growth increase observed for shoot height and shoot weight was 43 and 131%, respectively, the mean percentage growth increase for root weight was 177% (Table 2.1).

The ANOVA test showed significant treatment effects in 6 out of 9 corn growth variable measurements across all three trials. Significant differences

among bacterial strains, as indicated through the LSD test, occurred in 5 of the 6 measurements where a significant treatment effect occurred (Table 2.2). There was high inconsistency as to which strains were numerically ranked-within the top 3. Strains R181, R180 and R200 were found most often among the top 3 strains having FIT3 of 56, 50, and 50%, respectively, in all variable measurements. Strains R176, R190 and R198 did not appear among top 3 strains in any variable measurements. There was no significant difference among growth increase by the top 3 strains in most trials. The only one exception occurred for strain R181 that was significantly different from other strains for increasing root biomass in trial 1.

The best strains -R177, R180, R181 and R200 - from the sweet-corn growth promotion experiment based on highest GSF and FIT3 (Tables 2.1 and 2.2) were selected for further evaluation on wheat and soybean. Although *B. safensis* (R176) had a relatively high GSF (83%) as seen in Table 2.1, it was not selected because it did not appear among the top 3 strains in any growth variable measurement as seen in Table 2.2. *B. safeness* R173 instead, was selected to represent the species in the experiments on soybean and wheat.

2.3.2 Evaluation of five strains for growth promotion of soybean and wheat

The results from the soybean experiments, as indicated by Dunnett's test, showed that four strains - R173, R180, R181 and R200 - induced significant growth compared to control (Table 2.3). The bacterial strains stimulated growth at lower frequencies on soybean than that of corn experiments. Each of the strains

caused significant increases of multiple growth variables compared to the control in one or more trials of the experiment (Table 2.3). *B. safensis* strain R173 had the highest GSF of 63%, flowed by *B. simplex* R180 which had a next highest GSF percentage of 50% in all variable measurements. Strains R200 and R181 were less consistent for soybean growth promotion; having GSF percentages of 38 and 25%, respectively. Growth promotion was higher for the root growth than for shoot growth. Percent growth increase on root mostly exceeded 90%, whereas it was less than 50% for shoot growth (Table 2.3). None of the strains induced a significant increase in shoot height.

Analysis of variance (ANOVA) was conducted to evaluate the significance treatment effects. Significant treatment effects occurred in all 8 growth variable measurements (Table 2.3) but significant difference between bacterial strains occurred in 6 of those 8 cases, as indicated by LSD tests. Out of the four strains that were found among the top 2 strains category, R173, R180 and R181 were most frequently found in the category, FIT2 values of 100, 50, and 50% respectively, in all variable measurements. Strain R200 had a lower FIT2 value of 25%.

Among the five strains tested on wheat, three strains (R173, R181 and R200) significantly increased the growth of wheat compared to the control (Table 2.4). Each strain caused a significant increase of two or more growth variables in two or more trials compared to the control. Of the remaining two strains, R180 was less effective while R177 was ineffective for wheat growth promotion. Compared to the results on corn, strains R180 and R177 had lower GSF when

applied on wheat. Both R181, R200 and R173 had GSF values of 50, 50, and 25%, respectively. The GSF for strains R180 and R177 were 17 and 0%, respectively. Growth promotion was higher for root growth than for shoot growth. The mean for percentage growth increase for shoot height varied from 15 to 21%, shoot weight varied from 30 to 37% whereas that of root weight varied widely from 48 to 130% across all trials.

Analysis of variance (ANOVA) and LSD mean separation tests were conducted to identify significant treatment effects and to compare strains in each trial. Significant treatment effects were found in 5 out of 8 growth measurements across five trials (Table 2.4). Strains R181 and R200 were most consistently found among the top 2 strains, having a FIT2 percentages of 75 and 63%, respectively. Strains R173 and R180 had FIT2 percentage of 38 and 30%, respectively. In each of the five cases where significant growth increase occurred, there was no significant difference among the top 2 strains, as indicated by LSD tests.

These results showed that three strains (*B. safensis* R173, *B. simplex* R180 and *P. graminis* R200) were effective for promoting soybean growth, while four strains (*B. megaterium* R181, *B. safensis* R173 and *P. graminis* R200) were effective for promoting wheat growth as observed in greenhouse pot experiments. This indicated that these bacillus strains exhibited the potential for broad spectrum plant growth promotion effects.

2.4 Discussion

The first objective of this chapter was to determine which of the 12 bacillus strains has the potential to enhance plant growth using sweetcorn as the test plant. All the 12 strains significantly increased sweetcorn growth compared to control. Other studies have shown significant plant growth promotion effects on corn by large numbers of bacterial strains in greenhouse experiments. For example, 11 bacterial strains significantly increased different growth parameters including plant height, seed weight, seed per ear and leaf area on corn, after inoculation on corn seeds (Gholami et al., 2009).

The second objective was to test which of the top strains from the corn experiment can increase soybean and wheat growth in greenhouse pot experiments. R173, R181 and R200 were effective for both soybean and wheat growth promotion. *B. simplex* R180 was effective in soybean growth promotion but less effective for wheat growth promotion.

Hence, from all greenhouse experiments on corn, soybean and wheat, it is shown that four strains (*Bacillus safensis R173, B. simplex R180, B. megaterium R181* and *Paenibacillus graminis* R200 exhibit broad spectrum plant growth-promotion effect. Similar to these results, broad spectrum growth promotion effects have been reported by other authors. Ahmad et al. (2017) found that *B. subtilis* strain 330-2 induced significant growth stimulation of growth variables of both corn and rice plants compared to the controls in greenhouse pot experiments.

Tilak and Reddy (2006) reported that strains of *B. circulans* and *B. cereus* increased yields in maize (corn), wheat, and pigeonpea in field studies.

This study also showed that corn was more responsive to plant growth promotion effects by the strains compared to soybean and wheat. All the 12 strains significantly increased sweetcorn growth, whereas four out five strains significantly increased soybean and wheat growth. This type of result was observed in a study by Tilak and Reddy (2006), in which highest growth increase by Bacillus strains was observed on maize (corn) compared to wheat and pigeonpea. Other studies have shown limited wheat response to growth promotion by bacteria strains. For example, when Khalid et al. (2004) screened thirty bacterial strains for their plant growth promotion effects on wheat seedlings, only four isolates were found to be effective in plant growth promotion. These results support the present observation that corn was more responsive to growth stimulation by bacteria strains than wheat.

This study showed that *B. megaterium* R181 increased the growth of all the test crops consistently. Several previous reports have shown that strains of *B. megaterium* can increase the growth of different crop plants. Kaymak et al. (2008) reported that *B. megaterium* strain M3 improved different root growth parameters of inoculated mint cuttings compared to control treatments. In another study, the inoculation of *B. megaterium var. phosphaticum* resulted in growth promotion of pepper and cucumber plants compared to controls (Han and Lee 2006). Also, *B. megaterium* strain XTBG34 was shown to increase the growth of *Arabidopsis* by Zou et al., (2010). Another strain of *B. megaterium* promoted the growth and

development of bean (*Phaseolus vulgaris*) and *Arabidopsis thaliana* plant in studies conducted by López-Bucio et al., (2007). Furthermore, *B. megaterium* strain DE BARY increased plant growth after causing disease suppression in tea plants (Chakraborty et al., 2006). Also, treatment of apple seeds with a charcoal-based inoculant of *B. megaterium* significantly increased various growth attributes of six months old apple seedlings under nonsterilize soil conditions (Shirkot and Sharma, 2003).

The present study showed that *B. simplex* R180 was effective in increasing sweet-corn, soybean, and wheat growth. This species had not been reported to stimulate the growth of these crops. In previous studies, *B. simplex* was reported for growth promotion on kiwifruit (Erturk et al., 2010), pea plants (Schwartz et al., 2013), strawberry (Erturk et al., 2012) and tomato plants (Hassen and Labuschagne, 2010).

Paenibacillus graminis has not been reported for plant growth promotion by any author. However, the present study showed that *Paenibacillus graminis* R200 was effective in promoting the growth of sweet-corn, soybean and wheat. This is the first report of *P. graminis* for plant growth promotion activity. Several strains of *P. graminis* have been isolated from corn and wheat rhizospheres (el Zahar et al., 2008), and other strains exhibited certain plant growth promoting traits *in vitro* including nitrogen fixation ability and extracellular enzyme activities (Berge et al., 2002; Ding et al., 2005; Rodrigues et al., 2013). There was no any report, however, about the activity of the bacteria species for plant growth-promotion on any crop.

This study also found that *B. safensis* strains R173 and R176 have the potential to increase plant growth. Though several studies have isolated *B. safensis* and examined several strains for plant growth promotion traits (Damodaran et al., 2013; Singh et al., 2015), no other study has reported the species to be effective in plant growth promotion.

This study also showed that there were large variations in the level of growth promotion stimulated by strains from trial to trial. For instance, strains R181 and R180 induced higher growth on corn shoot than on root growth. Across all trials on corn, for R181 and R180, percent increase for shoot height varied from 18 to 45% and 30 to 41%, shoot weight varied from 40 to 140% and 68 to 118%, whereas percent increase for root weight ranged from 32 to 136% and 112 to 206%; respectively. The same trend of variation was also observed on soybean and wheat results. Variability in growth promotion by bacterial strains have been reported by authors. Mishra and Sundari (2013), observed similar variations among potential PGPR strains in different trials of greenhouse pot experiments.

The variability in plant growth-promotion by bacterial strains in these results might be due to changes in greenhouse environmental conditions such as temperature, resulting from seasonal changes in environmental temperature. Variability in plant growth promotion effects under relatively controlled greenhouse conditions have been observed by other authors. It is shown that plant growth promotion activity of bacteria strains can be influenced by factors such as soil indigenous organisms, soil organic content, root exudate components, soil

texture, pH, salinity, soil moisture and temperature (Cakmakçi et al., 2006; Banerjee et al., 2006; McSpadden-Gardener, 2004).

This study showed that 12 bacterial strains have potential to increase corn growth. Four out of the strains also exhibited broad spectrum plant growth promotion effects on three crops. *Bacillus safensis* (strains R173 and R176) and *P. graminis* (strain R200) were among the four strains with broad spectrum growth promotion effects. This is the first report showing that these endospore forming bacteria species exhibit plant growth promotion activity. However, plant growth promotion activity expressed by these strains varied from trial to trial. Field studies are required to further evaluate the strains' effectiveness for plant growth promotion in field production environments.

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2.6 Figures

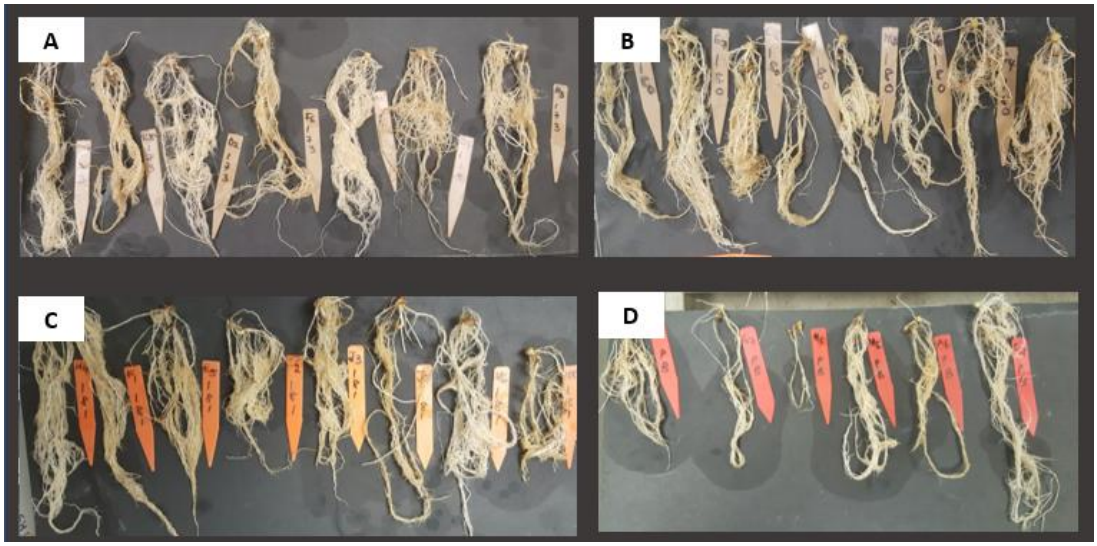

Figure 2.1. Growth promotion effects of bacillus strains on corn root growth in potted nonsterile soil. [A] *B. safensis* R173, [B] *B. simplex* R180, [C] *B. megaterium* R181, and [D] no-bacteria control

Table 2.1. Growth promotion effects of 12 Bacillus strains on sweetcorn in three trials of a greenhouse pot experiment.

Strain	% increase compared to control [a]									GSF (%) [b]
	Shoot height			Shoot fresh weight			Root fresh weight			
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	
Bacillus acidiceler R228	7	42*** [C]	28**	7	118***	66	-11	155***	92	44
B. megaterium R181	19***	45***	28**	40	140***	59**	36**	121***	132	78
B. megaterium R232	17	45***	12	24	144***	32**	-6	107***	173	44
B. pumilus R174	13	41***	7	30	126***	24	-3	117***	91	33
B. pumilus R183	12	40***	28**	33	103***	62	0	122***	12	44
B. pumilus R190	- [d]	38***	13	-	77***	32	-	93***	104	50
B. safensis R173	3	44***	15**	-15	137***	51**	-14	167***	222	56
B. safensis R176	-	34***	20**	-	111***	42**	-	124**	110	83
B. simplex R180	-	41***	30***	-	118**	68**	-	112***	206**	100
Lysinibacillus fusiformis R198	5	47***	17**	6	122***	33**	-25	135***	147**	56
Paenibacillus cineris R177	9	51***	20**	20	155***	42	3	168***	-8	67
P. graminis R200	-	54***	18**	-	215***	37	-	203***	75	67
Mean	18	43	23	-	131	48	36	135	177	NA[e]

a. Percentage increase of a growth variable by bacterial treatment compared to the control

b. GSF = Growth stimulation frequency; frequency at which a strain increased growth (at ≥ 95% confidence level) in all measurements across trials.

c. Asterisk (*) denotes significant difference between treatment and control measurements at 95 (**) and 99% (***) confidence levels, respectively, based on Dunnett's test.

d. Dash = No data because strain was not tested.

e. NA = Not applicable.

Table 2.2: Corn growth measurements as affected by treatment with 12 Bacillus strains in three trials of a greenhouse pot experiment.

Strain	Shoot height (cm)			Shoot fresh weight (g)			Root fresh weight (g)			FIT3
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	(%) [c]
Bacillus acidiceler R228	46	47ba [a]	40a	6.0	6.0bc	3.2ab	3.2b	3.5ab	0.9	22
B. megaterium R181	51	48ab	40a	8.0	6.5abc	3.0ab	4.9a	3.2ab	1.1	55
B. megaterium R232	50	48ab	35cde	7.0	6.6abc	2.5abcd	3.4b	3.1b	1.3	44
B. pumilus R174	49	47b	33cde	7.0	6.0bc	2.4bcd	3.5b	3.1b	0.9	11
B. pumilus R183	48	47b	40ab	7.0	5.5bc	3.1ab	3.6b	3.2ab	1.1	33
B. pumilus R190	-	46b	35bcde	-	4.8c	2.5abcd	-	2.8b	1.0	0
B. safensis R173	44	48ab	36abcd	6.0	6.4bc	2.9ab	3.1b	3.9ab	1.6	22
B. safensis R176	- [d]	47b	37abc	-	5.7bc	2.7abc	-	3.3ab	1.0	0
B. simplex R180	-	47b	41a	-	5.9bc	3.2a	-	3.2b	1.5	33
Lysinibacillus fusiformis R198	45	48ab	36abcd	6.0	6.0bc	2.5abcd	2.7b	3.5ab	1.2	0
Paenibacillus cineris R177	47	50ab	37abc	7.0	6.9ab	2.7abcd	3.7b	3.9ab	0.5	44
P. graminis R200	-	53a	37abcd	-	8.4a	2.6abcd	-	4.3a	0.9	50
Control	43	33c	31e	5	2.7d	1.9d	3.6b	1.5c	0.5	NA [e]
ANOVA P- value	0.0807	<.0001	0.0015	0.2245	0.0006	0.0419	0.0145	0.0052	0.614	NA

a. Numbers followed by the same letter in each column are not significantly different at α = 0.05 according to LSD test.

b. Asterisk (*): - significant difference between treatment and control measurements at 95 (**) and 99% (***) confidence levels respectively (Dunnett's test).

c. FIT3 (%): – Frequency in top 3 strains category as indicated by rank number. Green shade: rank number 1. Yellow shade: Rank number 2. Brown shade: Rank number 3.

d. Dash (-): - No data or strain was not tested.

e. NA = Not applicable

Table 2.3. Growth promotion effects of Bacillus strains on soybean plants in greenhouse pot experiments

Strain	Shoot fresh weight (g) and (% increase) [a]				Root fresh weight (g) and (% increase)				GSF (%) [d]	FIT2 (%) [e]
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 1	Trial 2	Trial 3	Trial 4		
Bacillus megaterium R181	1.3bc (18)	1.3ab (18)	3.7ab** (16)	3.8ab (3)	0.42b (-9)	0.64b (60)	1.7b*** (89)	2.1a (31)	25	50
B. safensis R173	1.5ab[b]** (36)	1.5a***[c] (36)	4.2a** (31)	4.2a (14)	0.62ab (35)	0.97a*** (142)	2.2a*** (144)	2.1a (31)	63	100
B. simplex R180	- [f]	-	3.9a** (22)	3.1b (-16)	-	-	2.2a*** (144)	1.3c (-19)	50	50
Paenibacillus cineris R177	1.1c (0)	1.1c (0)	-	-	0.38b (-17)	0.35c (-13)	-	-	0	0
P. graminis R200	1.6a*** (46)	1.2bc (9)	3.5ab (9)	3.6ab (-3)	0.88a** (91)	0.41c (3)	1.5b** (67)	1.8ab (13)	38	25
Control	1.1c	1.1bc	3.2ab	3.7b	0.46b	0.40c	0.9c	1.6bc	NA[g]	NA
ANOVA P-value	0.0004	0.0042	0.0543	0.0577	0.0034	<.0001	<.0001	0.0038	NA	NA

a. Percent (%) increase of a growth variable by bacterial treatment compared to the control
b. Numbers followed by the same letter in each column are not significantly different at α = 0.05 according to LSD test.
c. Asterisk (*) denotes significant difference between treatment and control measurements at 95 (**) and 99% (***) confidence levels, respectively, based on Dunnett's test.
d. GSF = Growth stimulation frequency; frequency at which a strain increased (at ≥ 95% confidence level) all growth variables across trials.
e. FIT2 = Frequency in top 2 strains category as indicated by rank number. Green shade: Ranked number 1 among treatments. Yellow shade: Ranked number 2 among treatments.
f. Dash (-) = No data because strain was not tested.
g. NA = Not applicable

Table 2.4: Growth promotion effects of Bacillus strains on wheat plants in greenhouse pot experiments. Growth measurements data for shoot height in trial 3 is not presented because ANOVA P > 0.10

Strain	Shoot height (cm) / (% increase) [a]		Shoot fresh weight (g)/ (% increase)			Root fresh weight/ (% increase)			GSF (%) [d]	FIT2 (%) [e]
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3		
Bacillus megaterium R181	38a** (15)	35** (21)	0.44a *** (47)	0.13 (30)	0.46 (7)	0.33a*** (154)	0.36a (29)	0.10a (43)	50	75
B. safensis R173	36ab [b] (9)	31 (7)	0.42a***[c] (40)	0.11 (10)	0.54 (26)	0.29ab** (123)	0.27ab (-4)	0.08ab (14)	25	38
B. simplex R180	37a (12)	33 (14)	0.31b (3)	0.13 (30)	0.59** (37)	-	-	0.07b (0)	17	33
Paenibacillus cineris R177	- [f]	-	-	-	-	0.20b (54)	0.24b (-14)	-	0	0
P. graminis R200	38a** (15)	33 (14)	0.43a*** (43)	0.13** (30)	0.49 (14)	0.28ab ** (115)	0.18b (-36)	0.11a** (57)	50	63
Control	33b	29	0.30b	0.10	0.43	0.13c	0.28ab	0.07b	NA[g]	NA
P-value	0.0599	0.1591	0.0011	0.0741	0.0934	0.005	0.018	0.0354	NA	NA

a. Percent (%) increase of a growth variable by bacterial treatment compared to the control
b. Numbers followed by the same letter in each column are not significantly different at $\alpha = 0.05$ according to LSD test.
c. Asterisk (*) denotes significant difference between treatment and control measurements at 95 (**) and 99% (***) confidence levels, respectively, based on Dunnett's test.
d. GSF = Growth stimulation frequency; frequency at which a strain increased (at $\geq$ 95% confidence level) all growth variables across trials.
e. FIT2 = Frequency in top 2 strains category as indicated by rank number. Green shade: Ranked number 1 among treatments. Yellow shade: Ranked number 2 among treatments.
f. Dash (-) = No data because strain was not tested.
g. NA = Not applicable

CHAPTER III

EVALUATION OF STRAINS FOR PLANT GROWTH-PROMOTING PHYSIOLOGICAL TRAITS *IN VITRO*

3.1 Introduction

Plant growth promoting rhizobacteria (PGPR) can increase plant growth via many direct or indirect mechanisms. Direct plant growth promotion occurs when PGPR increase plant growth in the absence of pathogens (Lugtenberg and Kamilova, 2009). Direct mechanisms may include the supply of nutrients for plant usage through several processes (Vessey, 2003). Examples of these processes may include the ability to fix atmospheric nitrogen (Lwin et al., 2012); solubilization of soil nutrients such as phosphate, potassium and sulfur, allowing for easier uptake by plants; and synthesis of siderophore to scavenge iron in iron-limited environment (Kafrawi, et al., 2014). As an example of plant growth promotion via nitrogen-fixation, *Bacillus* sp. strain SVPR30 was reported to fix a considerably high amount of nitrogen and increase rice root and shoot growth significantly compared to the controls (Beneduzi et al., 2008). Han and Lee (2006) demonstrated that the phosphate-solubilizing bacterium, *Bacillus megaterium var. phosphaticum,* increased photosynthesis rate and dry weight of inoculated plants compared to control plants. In another study, a phosphate solubilizing strain of *B. thuringensis* significantly increased the number of pods, pod weight, and seed yields of treated canola plants compared to the control (De Freitas et al. 1997).

Direct mechanisms also include production of plant growth regulators such as auxin, cytokinin and gibberellic acid to increase plant growth (Kafrawi, et

al., 2014; Lwin et al., 2012). Examples of plant growth increase via hormone production have also been documented. PGPR strain *B. amyloliqufaciens* FZB42, an indole acetic acid producer, was shown to promote plant growth in the presence of tryptophan, an indole acetic acid precursor (Idris et al., 2007). Similarly, strains of *B. pumillus* and *B. licheniformis* isolated from the rhizosphere of alder (*Alnus glutinosa*) were observed to produce high amounts of physiologically active gibberellins (Gutierez-Mañero et al., 2001); while the activity of cytokinin, another important plant hormone, was demonstrated in a PGPR strain of *P. polymyxa* (Timmusk et al., 1999).

Indirect growth promotion can occur when population densities and activities of plant pathogens and deleterious microorganisms are reduced by PGPR (Zablotowicz et al., 1991). It is related to the biocontrol of plant pathogenic organisms and deleterious rhizosphere microbes via mechanisms such as antibiotics and lytic enzyme production, competition for nutrients and niches within the rhizosphere, and induction of systemic resistance against pathogens (Bhattacharyya and Jha, 2012; Glick, 2012; Adesemoye and Egamberdieva, 2013; Lugtenberg and Kamilova, 2009).

Numerous examples of *Bacillus* strains with indirect plant growth promotion mechanisms have been reported. For example, *B. substilis s*train GB03 was observed to suppressed root rot disease of beans and increased dry weight and yields of treated plants significantly compared to control in greenhouse and field experiments (De Jensen et al., 2000). Xiang *et al.,* (2017) observed that *B. velezensis* strains Bve2 and Bve12 and *B. mojavensis* Bmo3 reduced the

population density of *Meloidogyne incognita*, the root-knot nematode and enhanced the growth of treated cotton plants compared to controls. In another study, *B. subtilis* strain ME488, was observed to suppress the growth of several plant pathogens tested *in vitro*, reduced the disease caused by *Fusarium* oxysporum on cucumber and *Phytophthora capsici* on pepper, and increased germination and seedling development compared to controls, when applied as a seed treatment on both plants in pot assays (Chung et al., 2008). Furthermore, application of surfactin produced by *B. amyloliqufaciens* KPS46 to soybean plants was found to inhibit *Xanthomonas axonopodis* pv. *glycines*, a bacterium causing pustule on soybean. The treatment also reduced the severity of bacterial pustule diseased and increased soybean growth (Preecha et al., 2010). In another study, *B. substilis* B28 producing protease, siderophore and hydrogen cyanide *in vitro*, was observed to reduce Fusarium wilt of chickpea, and significantly increased different growth parameters of chickpea plants including plant height and fresh and dry weight compared to controls in greenhouse experiments (Karimi et al., 2012).

In this study, the same twelve bacillus strains evaluated for plant growth promotion in pot experiments (Chapter 2) were assessed in the laboratory for physiological traits associated with direct and indirect growth promotion. Because all twelve strains exhibited some potential to promote plant growth on corn, it can be expected that there would be differences among strains as to the mechanisms involved. Testing of physiological traits can provide information as to the breadth of growth promotion mechanisms that can be expressed among the twelve strains.

It also was reported in Chapter 2 that the twelve bacillus strains could be separated into two groups (high efficacy and low efficacy) based on the level of growth promotion exhibited on corn, indicated by FIT3 and/or consistency of growth promotion, indicated by GSF, in repeated experiments. Another objective of evaluating the twelve strains for the physiological traits was to determine whether effective growth promotion could be predicted by a set of physiological traits or by expression of a high number of traits. Information as to the relationship between physiological traits and high growth promotion efficacy might be useful in developing screening strategies for effective PGPR strains.

3.2 Materials and methods

3.2.1 General procedures

The test organisms used in this study are summarized in Table 3.1. The test strains were isolated by Dr. Tony Adesemoye (University of Nebraska-Lincoln, West Central Research and Extension Center, North Platte) from the rhizosphere of wheat plants grown in Nebraska. The isolates of pathogenic bacteria, fungi, and oomycetes used in growth inhibition assays and the bacterial strains used as positive controls in various assays were provided either by Dr. Gary Yuen, University of Nebraska-Lincoln, or by Dr. Joseph Kloepper, Auburn University. All bacterial strains were stored at -75°C in storage broth. Bacterial strains were routinely cultured on 10% tryptic soy agar (TSA; Sigma Chemical, St. Louis) at 28 °C for 2 days. To produce cell suspensions, cells were harvested from culture plates with sterile spatula and suspended in sterile phosphate buffer

(PB) to 10^9 colony-forming units (CFU) ml^{-1}, with cell concentrations being determined turbidimetrically using a spectrophotometer at 600 nm.

3.2.2 Growth inhibition assay against plant pathogenic bacteria

Antagonism of the twelve *Bacillus* strains against three phytopathogenic bacteria (*Clavibacter michiganesis* subsp. *nebraskensis* (CMN), *Xanthomonas campestris* pv. *phaseoli* (XCP), and *Pectobacterium carotovorum* subsp. *carotovorum* (PCC) was evaluated by *in vitro* inhibition assays on 10% TSA and Nutrient Agar (NA) media. Cell suspensions were prepared for all bacterial strains as described above. Cultures were generated for each bacterial pathogen by evenly spreading 0.5 mL cell suspensions with a sterile spreader onto the surface of 10% TSA or NA plates. After the spread plates were air-dried aseptically in a transfer hood, five 3 mm diameter wells were made in each spread plate using a sterile cork-borer. Three wells were filled separately with 15 µL cell suspensions of three bacillus strains. The remaining two wells were filled with the same volume of a cell suspension of strain IN937a or sterile PB as positive and no-bacteria controls, respectively. The plates were left in the transfer hood for 15 minutes to allow absorption of the suspensions into the medium before incubation at 28°C for 2 days. Three replications were made for each plate. The observation of a clear halo zone around a well was an indication of antagonism activity by the test strain against the bacterial pathogen.

3.2.3 Growth inhibition assay against pathogenic fungi and oomycetes

Antagonism of the twelve bacillus strains against two phytopathogenic fungi (*Fusarium graminerum* and *Rhizoctonia solani)* and two oomycetes (*Pythium ultimum* and *P. irregulare)* was evaluated by *in vitro* inhibition assays on 10% TSA and PDA media. The center of each agar plate was inoculated with a 3-mm diameter fungal plug cut with a sterilized cork-borer from a 3 days old culture of a test fungus or oomycete. Each plate was co-inoculated with *Bacillus amyloliquefciens* KPS46 (positive control), sterile PB (negative control), and three test bacillus strains using sterile toothpicks onto five separate spots spaced equidistantly from the fungal plug. The test plates were incubated for 3 days at 25°C before they were examined for zones of hyphal growth inhibition around each bacterial colony.

3.2.4 Protease enzyme activity assay

The protease enzyme activity was evaluated on milk agar medium (Sigma Chemical, St. Louis) as modified by Dr. Tony Adesemoye's lab. The medium contained (g/L): powdered milk (10), yeast extract (0.5), ammonium sulfate (0.5), calcium chloride (0.5), potassium phosphate monobasic (0.1), potassium phosphate dibasic (0.1) and agar (18). Final pH was adjusted to 7.0 ± 0.2. Bacterial strains and *B. mojavensis AP-209* (positive control) were spot-inoculated onto separate spots on the test medium using a sterile toothpick. Three replications were made for each plate, and the test plates were incubated for 2 days at 28 °C. The presence of a clear halo zone around a bacterial colony indicated the presence of protease enzyme activity.

3.2.5 Chitinase enzyme activity assay

Bacterial strains were evaluated for chitinase enzyme activity on colloidal chitin medium (Abirami et al., 2016) containing (g/L): KH_2PO_4 (0.7), K_2HPO_4 (0.3), $MgSO_4.5H_2O$ (0.5), $FeSO_4.7H_2O$ (0.001), and ZnSO4 (0.001), $MnCl_2$ (0.001), colloidal chitin (5) and agar (20). The pH was adjusted to 7 ± 0.1. Loopfuls of test strains and *Lysobacter enzymogenes* C3 (positive control) were spot inocluated onto separate spots on the medium plate. Three replications were made for each plate. The plates were incubated at 28 °C for 5 days and observed for zone of clearing around bacterial colonies as indication for chitinase enzyme activity.

3.2.6 Assay for biosurfactant activity

The biosurfactant activity of the test bacterial strains was examined using the method described by Kobayashi and Yuen (2005). Briefly each strain was cultured for 2 days in tryptic soy broth (TSB) medium on a shaker (150 rpm) at room temperature, and the culture fluid was collected by centrifugation at 13,000 Xg for 15 minutes and filtration through 0.2 µm filters. Three 50 µL droplets of each filtrate were spotted onto the surface of parafilm. *Lysobacter enzymogenes* C3 and sterile TSB were used as positive and no-bacteria controls, respectively. The droplets were photographed after 15 minutes and the diameter of each droplet was measured. Spread of a droplet such that the droplet diameter was greater than

that of the no-bacteria control indicated presence of a biosurfactant. The experiment was performed three times.

3.2.7 Assay for siderophore production

Siderophore production was dectected using the Chrome Azurol S (CAS) siderophore assay (Schwyn and Neilands, 1987). The test bacterial strains were cultivated on iron deficient minimal salt medium (IDMSM) containing K_2SO_4 (1 g), Na_2HPO_4 (3 g), agar (15 g), CH_3COONH_4 (3 g), glucose (20 g), $MgSO_4.7H_2O$ (800 mg), $ZnSO_4.7H_2O$ (8.6 mg), MnSO4.H2O (0.113 mg) and arginine hydrochloride (1.5 mg). The final pH was adjusted to 7.1 ± 0.1. Each test strain was spot inoculated onto the center of the medium at one strain per plate using a sterile inoculation loop and incubated for 5 days at 28°C. Culture plates were flooded with 1 mL CAS solution, prepared as described by Louden et al. (2011). Plates inoculated with strain 94A-429 and sterile IDMSM plates were used as positive and negative controls respectively. Color change from blue to pink in the agar, under and around a bacterial colony within 30 minutes of applying the CAS solution was an indication of siderophore production by the bacterium.

3.2.8 Phosphate solubilization assay

Bacterial strains were evaluated for their ability to solubilize inorganic phosphate using Pikovskaya agar medium (Pikovskaya, 1948) containing calcium phosphate as the inorganic form of phosphate. The medium was composed of (g/L): yeast extract (0.5), glucose (10), calcium phosphate (5), ammonium sulfate

(0.5), potassium chloride (0.2), magnesium sulfate (0.1), manganese sulfate (0.0001), ferrous sulfate (0.0001), and agar (15). A loopful of each test strain and strain 94A-429 (positive control) was placed on two different spots on the medium plates and two replications were made for each plate. The plates were incubated at 28°C for 7 days. A zone of clearing around the colonies after 5 days was recorded as positive for phosphate solubilization.

3.2.9 Assay for indole acetic acid production

The bacterial strains were evaluated for their ability to produce indole acetic acid (IAA) using a tryptophan-supplemented agar medium and Salkowski's reagent (Salkowski, 1885; Gordon and Weber, 1951). Briefly, each bacterial strain was cultured in 10 mL 10% TSB for 1 day at 28°C. Then, 2 mL of the broth culture was transferred into 20 mL nutrient broth (NB) supplemented with L-tryptophan (0.5 g/L). Strain AP-282 with known indole acetic acid activity was used as the positive control. *Lysobacter enzymogenes* C3 and sterile NB were used as negative controls. The cultures were incubated at 28 °C for 6 days. Culture fluid supernatants were collected after centrifugation at 13000 X g for 15 minutes. The presence of IAA was determined by mixing 1 mL of bacterial culture supernatant, 2 mL Salkowski's reagent and 1 drop of orthophosphoric acid and incubating the mixture in the dark at room temperature for 30 minutes. Development of pink color in the reaction mixture indicated the presence of IAA. To quantify the amount of IAA produced, the absorbance of each reaction mixture

was measured using spectrophotometer at 530 nm and compared with a standard curve generated with an IAA dilution series.

3.2.10 Assay for nitrogen-fixation activity

The bacterial strains were evaluated for their nitrogen fixation ability on glucose nitrogen-free mineral (GNFM) agar medium with bromothymol blue (BTB) as an indicator (Ahmad et al., 2013). The medium composed of (g/L): glucose (10), dipotassium phosphate (1), magnesium sulfate (0.2), calcium carbonate (1), sodium chloride (0.2), sodium molybdate (0.005), and ferrous sulfate (0.1). Final pH was adjusted to 7.0 ± 0.2. The test strains and the positive control strain 99B-817 were inoculated onto the plates. Sterile plates were used as negative controls. Test plates were incubated at 28°C for 7 days and then flooded with BTB solution which was prepared by dissolving 0.5 g BTB into 100 mL distilled water and filter-sterilized. Color change in the agar from green to dark blue or bluish green was recorded as positive for nitrogen-fixation activity.

3.2.11 Growth pouch direct plant growth promotion assay

Bacterial strains were evaluated for their ability to directly increase the growth of sweetcorn (Cv. Sugar Buns f1 se+, Johnny's Selected Seeds), in a soil-less, semi-sterile environment (Figure 3.7). Seeds were surface-disinfected and treated as described in Chapter II. Seeds treated with sterile PB were used as the no-bacteria control. Treated seeds were sown into seed germination pouches (Mega International, United States) at 3 seeds per pouch. There were seven

replicate pouches for each treatment. The pouches were watered with 10 mL deionized tap water every other day and experiment kept at room temperature and 16/8 h light/dark hours for 10 days. At the end of the experiment, the shoots and roots were separated, and the shoot height, shoot fresh weight, total root length and numbers of lateral root were measured. The experiment was repeated three times. Dunnett's test ($\alpha = 0.05$) was used to determine whether a bacterial treatment was significantly different from the no-bacteria control. After analysis, a strain was recorded as positive for growth promotion if it increased the same growth variable in two or more trials or increased two or more growth parameters in the same trial.

3.3 Results

3.3.1 Growth inhibition assays against plant pathogenic microorganisms

Few of the test strains were inhibitory to either Gram-positive or Gram-negative plant pathogenic bacteria and none were inhibitory to both bacterial groups (Table 3.2). *B. pumilus* R183, but not the other two strains of *B. pumilus*, inhibited CMN on both 10% TSA and NA media, while *B. megaterium* R181 inhibited CMN only on NA medium. *B. pumilus* R190 was the only strain to inhibit the growth of XCP on NA medium (Table 3.2). None of the test strains was found to inhibit PC.

In the fungal growth inhibition assay, *B. megaterium* strain R181 and *B. pumilus* strains R174, R183, and R190 exhibited transitory inhibition of *Fusarium graminearum*, meaning that hyphal growth was slowed near the bacterial colony,

but the hyphae eventually grew through the bacterial colony. In contrast, growth inhibition zones around colonies of the positive control were unchanged in width throughout the experiment (Figure 3.1; Table 3.3). None of the test strains inhibited the growth of *Rhizoctonia solani* and the oomycetes *Pythium ultimum and P. irregulare* (data not shown).

These results indicate some strains have the potential for plant growth promotion via inhibition of specific groups of deleterious bacteria, but none of the test strains have a strong potential for indirect plant growth promotion via inhibition of fungal plant pathogens. In contrast to the results from this study, Yilmaz et al. (2006) reported that five *Bacillus* strains—two *B. brevis* and three *B. cereus*—were observed to inhibit the growth of different Gram-positive and Gram-negative bacteria *in vitro*, while, other authors (Jayaraj et al., 2005; Karimi et al., 2012; Patil et al., 2014) reported strong inhibition of fungi and oomycetes by strains of *B. subtilis*, which were not investigated in this study. The different results suggest that antimicrobial microbial activity varies among bacillus species and strains.

3.3.2 Protease enzyme activity assay

Some protease enzymes can hydrolyze the proteinaceous components of living microorganisms, and thus, be involved in indirect growth promotion. Protease also can be involved in direct growth promotion by mineralizing soil organic matter. The protease assay showed that nine out of the twelve test strains, produced protease enzymes, as indicated by the presence of clear halo zone

around the bacteria strains in the agar medium (Figure 3.2; Table 3.4). Three of the proteolytic strains (*B. pumilus* strains R183 and R190, and *B. simplex* R181) exhibited antibacterial activity in the bacterial growth inhibition tests, but the remaining six proteolytic strains were not inhibitory to bacteria (data not shown). This result could be related to the proteolytic enzymes produced by different bacillus species having greater or lesser activity on bacterial cell wall proteins. None of the strains of *Paenibacillus* and *Lysinibacillus* tested exhibited proteolytic activity. In contrast, Alvarez et al., (2006) reported strains of *P. peoriae* and *P. polymyxa* to produce extracellular protease in vitro, while Prabha et al., (2015) showed that a strain of *L. fusiformis* was positive for extracellular protease.

3.3.3 Chitinase enzyme activity assay

Chitinase is a hydrolytic enzyme that degrades chitin in the cell walls of true fungi and is produced by some plant growth promoting rhizobacteria (Seo et al., 2016). None of the test strains induced a clearing zone around its colony on colloidal chitin agar while the positive control strain, *Lysobacter enzymogenes* C3, induced a distinctive clearing zone on the medium (data not shown). This result indicated that none of the test strains has the potential for chitinase activity which corresponds to the strain exhibiting weak or no inhibition of fungal growth. The absence of chitinolytic activity contrasts with reports of *B. cereus* and *B. licheniformis* strains hydrolyzing colloidal chitin as a sole carbon source (Abirami et al., 2016; Pleban et al., 1997).

3.3.4 Assay for biosurfactant activity

Biosurfactant produced by some bacteria can be beneficial for plant growth via antimicrobial and biocontrol activity against plant pathogens (de Bruijn *et al.*, 2007; Raaijmakers *et al.*, 2010; De Souza et al., 2003). Biosurfactant activity was accessed by examining culture supernatant spread on a hydrophobic surface, as measured by diameters of supernatant droplets. This activity was expressed by all *B. pumilus* strains and both *B. safensis* strains as indicated by significantly wider supernatant droplet compared to the negative control (Table 3.5). Biosurfactant production by strains R183 and R190 might contribute to their ability to inhibit the growth of bacteria observed in the inhibition assay.

3.3.5 Assay for siderophore production

Siderophores are low molecular weight compounds produced by some bacteria to bind and acquire ferric iron nutrient in iron-deficient environments. The production of siderophores by PGPR can be involved in indirect plant growth promotion via suppression of pathogens via ferric iron competition and in direct plant growth promotion by increasing iron availability to plants. Four of the bacillus strains (R180, R181, R190 and R232) were positive for siderophore production on CAS agar medium, as indicated by a blue to pink color change in the medium (Figure 3.3; Table 3.6). These results indicated that the four strains have the potential to increase plant growth directly and/or indirectly via siderophore. Santos et al. (2014) also detected siderophore production by a *B.*

megaterium strain in an iron-deficient medium, while Chaiharn et al., (2009) found 23% of their bacterial strains (compared to 33% in this study) produced siderophore in vitro.

3.3.6 Phosphate solubilization assay

Phosphorus (P) is an important plant nutrient in soil. However, it mostly occurrs in soil in the form of complex phosphate compounds, thereby not readily available for plants and microbes. Some PGPR can make more P available by solubilizing phosphate complexes into forms which can be easily assimilated by both to plants and microbe. In this study strains R173, R177, R181, and R232 exhibited phosphate solubilization on Pikovskaya's agar medium, as indicated by the presence of clearing halo zone around the bacterial colony on the medium (Figure 3.4; Table 3.7). In a similar study, Wang et al., (2017) found that *B. cereus* strain YL6 solubilized inorganic phosphate on growth medium, and several strains of *Paenibacillus species* were found by Marra et al., (2012) to solubilize inorganic phosphate.

3.3.7 Assay for indole acetic acid production

Indole acetic acid is an important plant growth regulator that promote cell division, stem and root growth. Important roles of IAA in the development of the plant root system and induction of plant growth promotion have been demonstrated (Patten and Glick, 2002). All the bacterial strains were evaluated for their ability to produce IAA in nutrient broth supplemented with tryptophan.

Seven strains - R228, R232, R181, R176, R173, R198, and R177 - produced indole acetic acid, as indicated by supernatant color change from yellow to pink in Salkowski's reagent (Figure 3.5; Table 3.8). These strains demonstrated the potential to increase plant growth by producing indole acetic acid in the rhizosphere. Similar observation of IAA production by *Bacillus* strains has been reported by other authors, for example, *B. amyloliquefaciens* strain FZB42 (Idris, et al., 2004), seven *Bacillus* species (Kumar, et al. 2012), and several *Bacillus* strains (Beneduzi et al., 2008).

3.3.8 Assay for nitrogen-fixation activity

Some PGPRs increase plant health and induced plant growth promotion by fixing atmospheric nitrogen into the rhizospheric soil. None of the test strains was positive for nitrogen-fixation (data not shown), as shown by lack of color change in bacterial culture plate compared to the positive control strain which induced a color change from green to blue green color. In contrast to this study, nitrogen-fixing ability have been reported for several bacillus strains by other authors. For example, many bacillus strains were scored positive for nitrogen-fixation ability in a study by Seldin et al., (1984), while three *Bacillus* strains, CNPSo 2476, CNPSo 247, and CNPSo were positive for nitrogen-fixation ability in a study conducted by Szilagyi-Zecchin et al., (2014).

3.3.9 Growth pouch direct plant growth promotion assay

The strains were evaluated for their ability to increase sweetcorn growth in growth pouches. Because growth pouches were maintained in semi-sterile conditions, the experiment can be considered as a general test for direct growth promotion. Nine of the twelve strains exhibited the potential to increase plant growth in growth pouches, increasing at least one growth parameter (lateral root number, root length, shoot height and shoot weight) compared to the control in at least two out of three trials or measuring multiple growth parameters in a single trial (Figure 3.6; Table 3.9).

3.4 Discussion

The results from the 10 physiological trait tests are summarized in Table 3.10 for the twelve bacillus strains, the strains being ordered according to species names. The ability to promote corn growth in growth pouches, which indicates direct growth promotion activity, was a common trait to most of the strains, but nitrogen-fixation is not involved in direct growth promotion by any of the strains. Expression of all other traits, however, appears to differ considerably among strains. Differences exist among strains of the same species. Although the differences are evident within those species represented by multiple strains, *B. pumilus* strains might be involved in growth promotion via indirect mechanisms by antagonizing deleterious microbes. All the *B. pumilus* strains exhibited protease, biosurfactant, siderophore activities, and inhibition of fungal and bacterial growth *in vitro,* which are traits associated with antagonism. Only one

strain, *B. pumilus* R183, promoted corn growth in growth pouches. Except for proteolysis, the strain did not exhibit specific traits associated with direct growth promotion that would explain its activity in growth pouches. Other than the strains of *B. pumilus* and *B. megaterium* strain R181, no strains exhibited clear indication that antagonism could be involved in growth promotion. Nearly all non-antagonist strains displayed proteolysis, siderophore production, phosphate utilization, IAA production, or combinations. These traits support the conclusion that the strains might be active via direct growth promotion. The sole exception is *P. graminis* strain R200, which did not express any of the in vitro traits.

The ability of a strain to express a particular trait *in vitro* does not mean that the strain can express that trait when it is inhabiting the rhizosphere. Considerably more research is required to prove that any of these traits are mechanisms involved in promoting plant growth as exhibited in Chapter 2. Furthermore, the selection of traits tested in this study does not represent all the traits associated with plant growth promotion, as indicated by the results with *P. graminis* R200.

Another objective in testing the twelve bacillus strains was to determine whether there was a relationship between the expression of certain *in vitro* traits and high growth promotion efficacy as observed in Chapter II. Information given in Table 3.10 is presented in Table 3.11 with the strains being grouped into the high efficacy and low efficacy groups. Clearly there are no individual traits or a pattern of traits that distinguish the high efficacy group from the low efficacy group. Indole acetic acid production and phosphate solubilization were the traits

found more common among strains in the high efficacy group than strains in the low efficacy group. Thus, it is possible that the traits might contribute to high plant growth-promotion effectiveness. But since the traits were absent from two high efficacy strains (R180 and R200), therefore, they are not the determinants for high growth promotion efficacy. Other traits - protease, siderophore, biosurfactant and antimicrobial inhibition - were found more commonly among the low efficacy strains than the high efficacy strains (Table 3.11).

The relationship between physiological traits and effectiveness in growth promotion also was examined from the perspective of numbers of physiological traits associated with high and low efficacy. Expression of numerous traits by a strain was not always consistent with exhibition of high plant growth-promotion efficacy by strains. For example, *B. pumilus* R190, which was positive for 6 out 10 traits, was in the low efficacy category in term of plant growth-promotion efficacy. The converse also was true, as shown by *P. graminis* R200. This strain in the high efficacy group, exhibited no physiological trait other than the ability to promote growth in a growth pouch.

My main conclusion is that effectiveness of a PGPR strain in promoting plant growth in a soil environment cannot be predicted by physiological traits alone. While having the ability to express mechanisms that lead to growth promotion is a requirement, other factors, such as the capacity for aggressive rhizosphere colonization, would also be important to efficacy. Given that physiological traits are not predictive of effectiveness in growth promotion, I would not suggest that testing of physiological traits should be the primary

method for the screening of bacteria for effective plant growth promoters. While testing for direct traits such as phosphate solubilization and IAA production might lead to identification of effective strains, the tests might also exclude some effective candidates, such as R180 and R200 in this study. Greenhouse pot tests, such as those described in Chapter 2, are the simplest and most direct screening method to identify effective strains. Another advantage of using pot tests is that effective growth promoter strains coming from pot tests can be presumed to be effective in rhizosphere colonization and in expressing growth promotion mechanisms in the rhizosphere.

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3.6 Figures

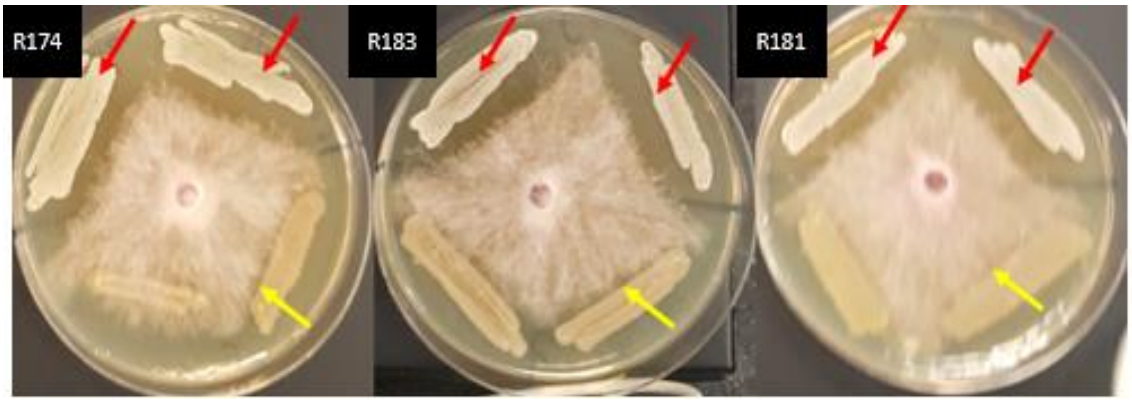


Figure 3.1. Antagonism activity (yellow arrow) of *B. pumilus* strains (R174 and R183), and *B. megaterium* R181 against *F. graminearum. Red arrow:* Positive control strain KPS46

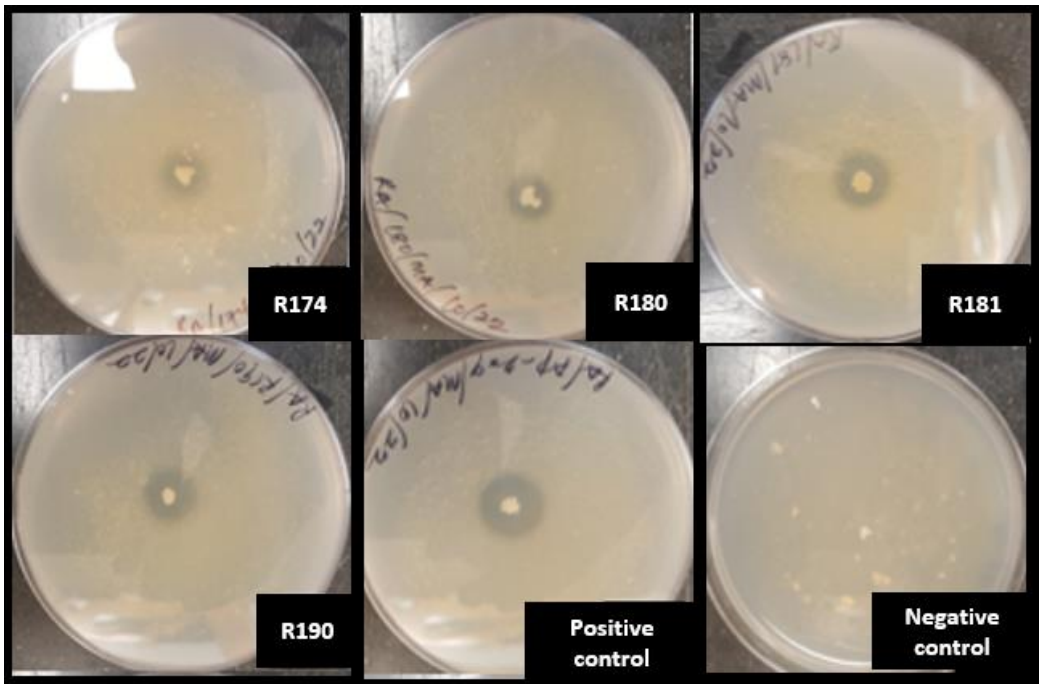


Figure 3.2. Proteolytic enzyme activity of representative bacillus strains on Milk agar medium.

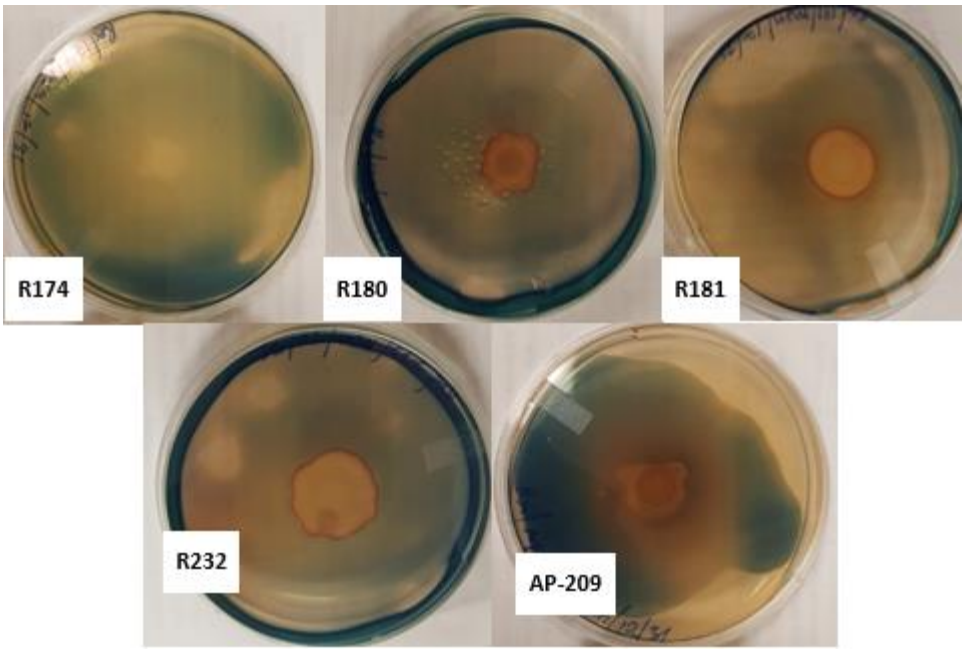


Figure 3.3. Siderophore production by *Bacillus simplex* R180, *B. megaterium* strains R181 and R232, and strain AP-209 (positive control) indicated by presence of pink color around bacterial colonies. *B. safensis* strain R174 did not exhibit siderophore production.

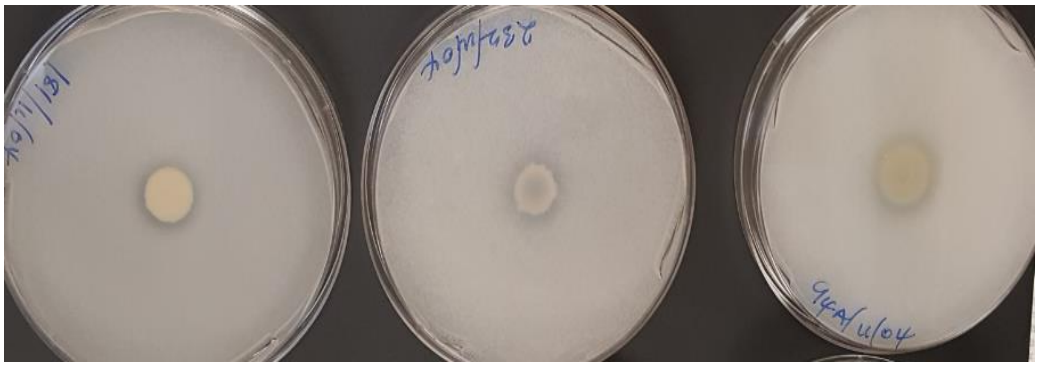


Figure 3.4. Phosphate solubilization activity of Bacillus strains: (Left to right) *B. megaterium* R181 and R232, and 94A-429 (positive control).

Figure 3.5. Production of indole acetic acid (Pink colored bottles) by bacterial strains. AP-282 = Positive control. Nutrient Broth (NB) and C3 = negative control.

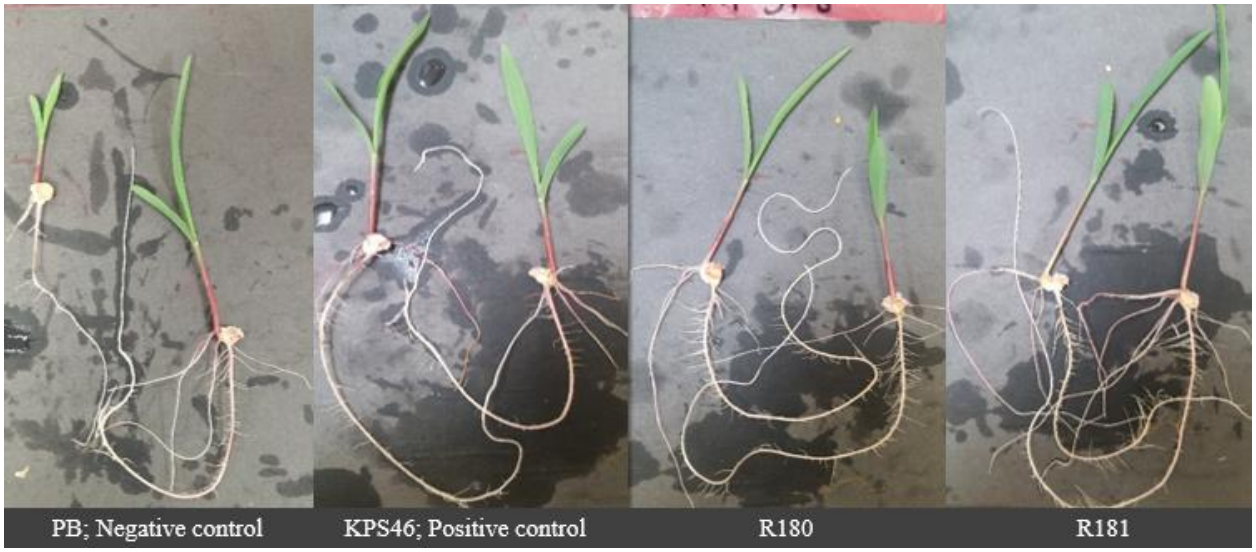


Figure 3.6. Effects of bacillus strains on corn growth in growth pouches. Strains increased corn seedling growth including lateral root number compared to negative control

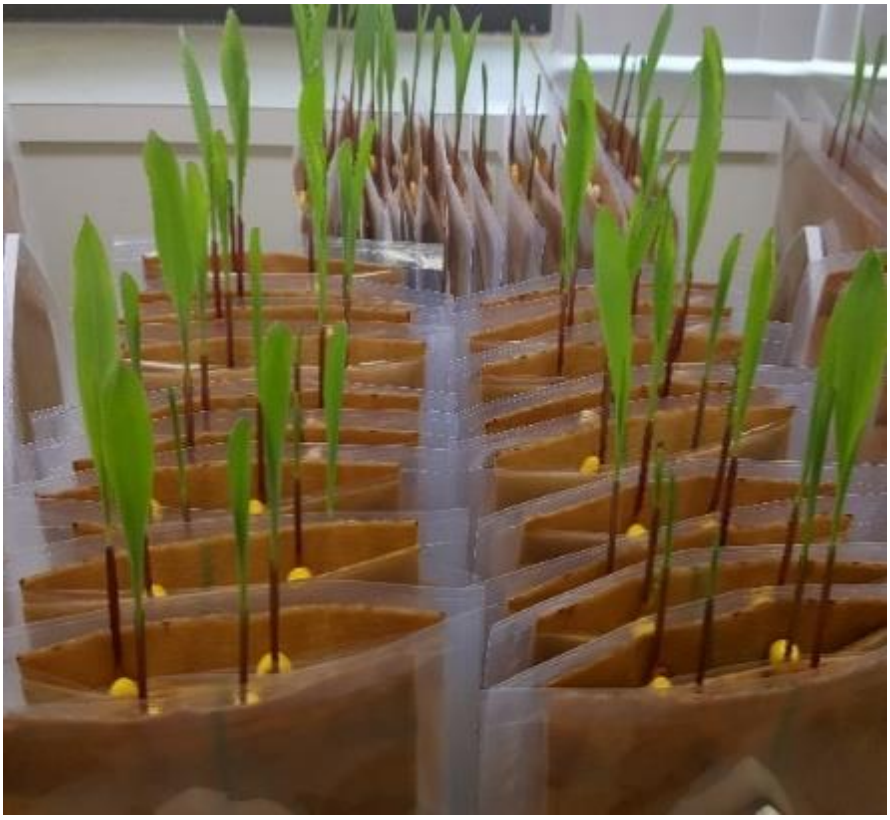


Figure 3.7. Growth pouch assay for assessing direct promotion of sweetcorn growth by bacillus strains

3.7 Tables

Table 3.1. List of all organisms - 12 test *Bacillus strains*; seven bacterial strains (positive controls); three pathogenic bacteria, two fungi, and two oomycetes (challenge organisms) used *in vitro* assays.

Test organism	Purpose /characteristics/ Accession number	Source
Bacillus acidicelerR228	Test strain/KY515411	Tony Adesemoye University of Nebraska-Lincoln.
B. megateriumR181	Test strain/KY807994	
B. megateriumR232	Test strain/KY515414	
B. safensis R176	Test strain/KY515395	
B. safensis R173	Test strain/KY515393	
B. simplex R180	Test strain/KY515398	
B. pumilus R174	Test strain/KY515394	
B. pumilusR183	Test strain/KY515399	
B. pumilus R190	Test strain/KY515404	
Lysinibacilus fusiformisR198	Test strain/KY515408	
Paenibacillus cineris R177	Test strain/KY515396	
P. graminisR200	Test strain/KY515409	
B. amyloliquefaciens KPS46	Positive control for fungi inhibition assay	Gary Yuen University of Nebraska-Lincoln
Lysobacter enzymogenes C3R5	Positive control for biosurfactant and chitinase activity.	
B. subtilis amyloliquefaciens IN937A	Positive control for bacteria inhibition assay	
B. mojavensis AP-209	Positive control for protease enzyme activity	Joseph Kloepper Auburn University, Alabama.
Lysinibacillus macrolides AP-282	Positive control for IAA assay.	
Gram negative strain 94A-429	Positive control for siderophore and phosphate solubilization.	
Gram positive strain 99B-817	Positive control for nitrogen-fixation assay.	
Clavibacter michiganensis susp. *nebraskensis* (CMN)	Plant pathogenic bacteria for inhibition assay	Gary Yuen University of Nebraska-Lincoln
Pectobacterium carotovorum (PC)		
Xanthomonas campestris pv. *campestris* (XCP)		
Rhizotonia solani R251	Plant pathogenic fungi for inhibition assay	
Fusarium graminearium PH-1		
Pythium ultimum	Plant pathogenic oomycetes for inhibition assay	
Pythium irregulare		

Table 3.2: Assessment of bacillus strains for antagonism against Gram-positive and Gram-negative bacteria - *Clavibacter michiganensis* subsp. *nebraskensis* (CMN), *Xanthomonas campestris* (XCP) and *Pectobacterium carotovorum* (PC)

Strain	10% Tryptic soy agar			100% Nutrient agar		
	CMN	XCP	PC	CMN	XCP	PC
Bacillus acidiceler R228	-	-	-	-	-	-
B. megaterium R181	-	-	-	+	-	-
B. megaterium R232	-	-	-	-	-	-
B. pumilus R174	-	-	-	-	-	-
B. pumilus R183	+	-	-	+	-	-
B. pumilus R190	-	-	-	-	+	-
B. safensis R173	-	-	-	-	-	-
B. safensis R176	-	-	-	-	-	-
B. simplex R180	-	-	-	-	-	-
Lysinibacillus fusiformis R198	-	-	-	-	-	-
Paenibacillus cineris R177	-	-	-	-	-	-
P. graminis R200	-	-	-	-	-	-
IN37a (Positive control)	-	-	-	-	+	+

Plus sign (+) = Inhibition zone found around bacterial well.
Minus sign (-) = No inhibition zone around bacterial colony.

Table 3.3. Assessment of bacillus strains antagonism against *Fusarium graminearum*

Strains	10% TSA	100% TSA	10% PDA	100% PDA
Bacillus acidiceler R228	-	-	-	-
B. megaterium R181	Tr	Tr	Tr	-
B. megaterium R232	-	-	-	-
B. pumilus R174	Tr	Tr	Tr	Tr
B. pumilus R183	-	Tr	Tr	Tr
B. pumilus R190	Tr	Tr	Tr	Tr
B. safensis R173	-	-	-	-
B. safensis R176	-	-	-	-
B. simplex R180	-	-	-	-
Lysinibacillus fusiformis R198	-	-	-	-
Paenibacillus cineris R177	-	-	-	-
P. graminis R200	-	-	-	-
KPS46 (Positive control)	+	+	+	+
Negative control	-	-	-	-

Plus Sign (+) = Inhibition zone around bacterial colony did not change over time.
Minus sign (-) = No inhibition zone around bacterial colony.
Tr = Transitory inhibition; hyphae eventually overgrow bacterial colony.

Table 3.4. Assessment of bacillus strains for protease enzyme activity

Strain	Protease activity
Bacillus acidiceler R228	+
B. megaterium R181	+
B. megaterium R232	+
B. pumilus R174	+
B. pumilus R183	+
B. pumilus R190	+
B. safensis R173	+
B. safensis R176	+
B. simplex R180	+
Lysinibacillus fusiformis R198	-
Paenibacillus cineris R177	-
P. graminis R200	-
B. mojavensis AP-209 Positive control	+

Plus Sign (+) = Protease present. Minus Sign (-) = Protease absent

Table 3.5. Assessment of bacillus strains for biosurfactant production activity as indicated by spread of culture fluid droplet on prarfilm.

Strain	Culture fluid droplet diameter (cm)
Bacillus acidiceler R228	0.56
B. megaterium R181	0.54
B. megaterium R232	0.56
B. pumilus R174	0.64***
B. pumilus R183	0.66***
B. pumilus R190	0.60***
B. safensis R173	0.60***
B. safensis R176	0.61***
B. simplex R180	0.55
Lysinibacillus fusiformis R198	0.55
Paenibacillus cineris R177	0.55
P. graminis R200	0.55
Lysobacter enzymogenes C3 (Positive control)	0.64***
Broth (Negative control)	0.52

Asterisks (***) denotes significant difference between treatment and negative control at 99% confidence level as determined by Dunnett's test.

Table 3.6. Assessment of bacillus strains for siderophore production

Strain	Siderophore activity
Bacillus acidiceler R228	-
B. megaterium R181	+
B. megaterium R232	+
B. pumilus R174	-
B. pumilus R183	-
B. pumilus R190	+
B. safensis R173	-
B. safensis R176	-
B. simplex R180	+
Lysinibacillus fusiformis R198	-
Paenibacillus cineris R177	-
P. graminis R200	-
Gram negative bacterial strain 94A-429	+

Plus Sign (+) = Siderophore present.
Minus Sign (-) = Siderophore absent

Table 3.7. Assessment of bacillus strains for phosphate solubilization activity

Strain	Phosphate solubilization
Bacillus acidiceler R228	-
B. megaterium R181	+
B. megaterium R232	+
B. pumilus R174	-
B. pumilus R183	-
B. pumilus R190	-
B. safensis R173	+
B. safensis R176	-
B. simplex R180	-
Lysinibacillus fusiformis R198	-
Paenibacillus cineris R177	+
P. graminis R200	-
Gram negative bacterial strain 94A-429	+

Plus Sign (+) = Phosphate solubilization activity present.
Minus Sign (-) = Phosphate solubilization activity absent

Table 3.8. Assessment of bacillus strains for indole acetic acid production

Strain	Indole acetic acid
Bacillus acidiceler R228	+
B. megaterium R181	+
B. megaterium R232	+
B. pumilus R174	-
B. pumilus R183	-
B. pumilus R190	-
B. safensis R173	+
B. safensis R176	+
B. simplex R180	-
Lysinibacillus fusiformis R198	+
Paenibacillus cineris R177	+
P. graminis R200	-
L. macrolides AP-282 (positive control)	+

Plus Sign (+) = Indole acetic acid present.
Minus sign (-) = Indole acetic acid absent

Table 3.9. Assessment of effects of bacillus strains on sweetcorn growth in growth pouch experiments

Strain	% increase compared to control [a]									
	Lateral root number			Root length (cm)			Shoot height (cm)		Shoot fresh weight (g)	
	T1[b]	T2	T3	T1	T2	T3	T2	T3	T2	T3
Bacillus acidiceler R228	17	20	0	27	15**[C]	10	44***	18**	40***	20
B. megaterium R181	50**	20	-10	41**	-17	8	0	13	0	20
B. megaterium R232	17	40**	-10	23	2	4	39**	9	40**	20
B. pumilus R174	33**	20**	-10	37**	2	10	28***	23**	20	20
B. pumilus R183	17	-40	-20	46**	-17	-4	-6	-9	-20	0
B. pumilus R190	-17	-20	-10	10	-4	-2	17	-5	20	0
B. safensis R173	-17	0	0	23	-18	8	-11	27**	-20	20
B. safensis R176	-50	40**	-10	-10	-17	10	22**	18	20	20
B. simplex R180	17	0	-10	46**	-7	14	22**	13	20	20
Lysinibacillus fusiformis R198	33**	-20	-10	18	-10	20**	28***	32***	20	40**
Paenibacillus cineris R177	33**	-20	-10	37**	-13	2	11	13	0	20
P. graminis R200	33**	0	0	41**	-15	14	22***	18	40**	20

a. % increase was calculated using the equation $\frac{Mt-Mc}{Mc} X\ 100$, where Mt and Mc are the mean measurements of the treatment and control, respectively.
b. T1-T3 = Trial 1 to trial 3.
c. "**" and "***" denote significant difference between treatment and control measurements at 95 and 99% confidence levels, respectively, as determined by Dunnett's test.

Table 3.10. Summary of plant growth-promoting physiological traits of test bacillus strains as exhibited in *in vitro* assays.

Strain	Antif	Antib	Pro	Chi	Bios	Sid	Phos	IAA	N2 fixation	Pouch assay
Bacillus acidiceler R228	-	-	+	-	-	-	-	+	-	+
B. megaterium R181	+	+	+	-	-	+	+	+	-	+
B. megaterium R232	-	-	+	-	-	+	+	+	-	+
B. pumilus R174	+	-	+	-	+	-	-	-	-	-
B. pumilus R183	+	+	+	-	+	-	-	-	-	+
B. pumilus R190	+	+	+	-	+	+	-	-	-	-
B. safensis R173	-	-	+	-	-	-	+	+	-	-
B. safensis R176	-	-	+	-	+	-	-	+	-	+
B. simplex R180	-	-	+	-	+	+	-	-	-	+
Lysinibacillus fusiformis R198	-	-	-	-	-	-	-	+	-	+
Paenibacillus cineris R177	-	-	-	-	-	-	+	+	-	+
P. graminis R200	-	-	-	-	-	-	-	-	-	+
Positive control	+	+	+	+	+	+	+	+	+	+
Negative control	-	-	-	-	-	-	-	-	-	-

Antif: - Antifungal, Antib: - Antibacterial, Pro: - Protease, Chi: - Chitinase, Bios: - Biosurfactant, IAA: - Indole acetic acid, $N_{2:}$ - Nitrogen.
Plus Sign (+) = Trait present. Minus sign (-) =Trait absent

Table 3.11. Diversity of traits and plant growth promotion efficiency of bacillus strains on corn

Strain	Antif	Antib	Pro	Chi	Bios	Sid	Phos	IAA	N2 fixation	Pouch assay	Efficacy on corn.
Bacillus megaterium R181	■	■	■			■	■	■		■	
B. safensis R173			■				■	■			
B. safensis R176			■		■			■		■	High
B. simplex R180			■		■	■				■	
Paenibacillus cineris R177							■	■		■	
P. graminis R200										■	
B. acidiceler R228			■					■		■	
B. megaterium R232			■			■	■	■		■	
B. pumilus R174	■		■		■						
B. pumilus R183	■	■	■		■					■	Low
B. pumilus R190	■	■	■		■	■					
Lysinibacillus fusiformis R198	■	■	■		■	■		■		■	

Antif: - Antifungal, Antib: - Antibacterial, Pro: - Protease, Chi: - Chitinase, Bios. Biosurfactant, IAA: - Indole acetic acid, $N_{2:}$ - Nitrogen.
Black shade = Trait present. White shade = Trait absent

CHAPTER IV

CLOSING: LOOKING BACK, LOOKING FORWARD.

In this concluding chapter, I want to restate my views; what I have learned in the process of this research. The information I provide here is not necessarily directed to only the scientific community but also to any reader who might be interested in the practical use of PGPR for promoting plant growth. First, I present the objectives of the studies and itemize their corresponding findings. Then, I present some tips that might be helpful as a guide for students who might need to conduct studies of this nature for the first time. Also, I make some recommendations regarding the screening procedures for potential PGPR and make some statements about the use of PGPR in environments where there is no access to sophisticated research facilities and technologies such as in underdeveloped and developing countries. Then on a final note, I make some suggestions as to what research experiments can be conducted in field or greenhouse to further examine the potentials of the PGPR strains found in this study.

At the beginning of this study, I set out to provide answers to four specific objectives. The first objective was to assess the growth promotion potentials of twelve bacillus strains on sweetcorn *in vivo.* Using greenhouse pot experiments, I found that eleven out of twelve strains increased sweetcorn growth at least in two out of three trials. The growth promotion effects were relatively high; over 200% compared to controls in some cases, but the level of growth stimulation varied among strains (Table 2.1). The second objective was to identify strains that can

exhibit broad spectrum growth promotion, i.e. increase growth of soybean and wheat. On soybean, four out of five strains increased growth significantly compared to control. Root growth stimulations were as high as 144%, compared to controls (table 2.3). On wheat, three out of five strains increased growth significantly compared to controls. Root growth stimulations were as high as 154% over the controls (table 2.4). These results suggest that bacillus strains exhibit great potential for increasing plant growth. Also, as seen from the result, response of crop plant types to growth promotion effects varied from one crop to another. This might suggest that while a PGPR product is effective, it does not guarantee increased growth across different crop types. These findings might provide the reasons for the inconsistent performance often reported for many commercial PGPR products. The results also showed that root growth was more sensitive to growth stimulation effects than shoot growth across all the test crops. This may suggest that the strains might be more effective if applied on root or tuber crops such as potato; however, this hypothesis has yet to be tested.

The third specific objective was to assess the potential mechanisms of action for each strain—to know which of the strains increase plant growth directly via supply of plant nutrients and hormones to plants or indirectly through antagonism against deleterious microbes. After conducting *in vitro* assays, I found that most of the strains exhibited both direct and indirect mechanisms, but direct mechanisms were more common among the strains. This was shown in the growth pouch assay test results (Table 3.9). Most of the strains also exhibited traits such as siderophore production, phosphate utilization, IAA production, or

combinations of the traits that are indicated for direct mechanism. Although some of the strains displayed antagonism— an indirect mechanism trait—, the trait was not expressed by most of strains. This might suggest that bacillus strains express traits associated with direct mechanisms more easily than indirect mechanisms as supported by the fact that direct mechanisms were exhibited by most of the bacillus strains in this study.

The fourth specific objective was to determine if possession of a specific set of traits or numerous traits will result in highly efficient plant growth-promotion. The *in vitro* assays showed that IAA production and phosphate solubilization activity were commonly found together in high efficient strains for growth promotion in greenhouse pot tests. In three out of four cases, the traits were both found in high efficient strains, but they were not found in strains R180 and R200 which were also in the high efficient group. This implies that the presence of both traits might have contributed to the high growth-promotion efficiency shown by the strains, but they were not the only traits responsible for the high growth-promotion efficiency. As a conclusion, I will say that although certain traits may contribute to high PGPR effectiveness, no specific combination of traits can always guarantee high PGPR effectiveness. Except a trait is always found with every incidence of high plant growth-promotion efficiency exhibited by a PGPR strain, such trait(s) should not be attributed for high PGPR efficacy. Likewise, I found that numerous *in vitro* traits were not always responsible for high plant growth promotion activity. A strain (*P. graminis* R200) that tested positive in only one *in vitro* assay increased plant growth with high efficiency

compared to some other strains (R183, R190 and R232) that exhibited many traits *in vitro* but which increased plant growth with low efficiency. This implies that the presence of numerous *in vitro* traits in a strain does not give assurance that such strain will exhibit high efficiency in plant-growth promotion in soil systems.

Besides the findings of this research, I would like to point out some tips I found helpful during these studies. The tips may be helpful for individuals or students who might need to perform any study of this nature for the first time. One important thing to note down is this: conducting any kind of research experiments including laboratory and greenhouse experiments will require one to first obtain relevant information and knowledge about the research; know the kinds of experiments to be conducted, and study how each experiment is conducted before starting the real experiments. The next step is to conduct small-sized preliminary tests as a prototype of the experiments to assist in getting used to the procedures and technologies involved in the experiments. It would also help in deciding what final procedure would work best for a specific experiment. As for me in this study, as the first step, I started with reviewing research publications, searching through similar studies to obtain relevant idea for developing my procedures. Then, as the second step, I had to perform several preliminary tests before I was able to get acclimatize to the procedures and decide on the most appropriate specific techniques. In particular, I did preliminary tests before deciding the most appropriate seed-soaking time, soil-mix ratio, seeding rate, negative controls—choosing between phosphate buffer and sterile distilled water—, and when to carry out data collection.

Furthermore, I would like to discuss the drawback and limitations of the techniques used in this study. The growth pouch experiment was conducted on a shelf platform in the lab. So, some pouches became contaminated with saprophytic fungi and the mycelium growing association with corn seedlings the pouches complicated the collection of root data. Therefore, conducting such experiment in a more sterile environment such as growth chamber would be something I would do to avoid such incidence. Also, I might use another method such as the agar plate assay technique which can provide a better substrate for assaying seedling growth compared to the pouch assays which easily dries out most times. Besides these, in the greenhouse study, plant growth-promotion effects of the strains might be improved if their cell suspensions were applied into the soil mixture rather than just as seed treatments. Also, in some cases, soaking of seeds (such as soybean seeds) in bacterial cell suspensions for up to certain time often resulted to seeds having less germination vigor. These observations are something I would adjust if I were to perform this same study again.

As to my recommendations based on the findings of these studies, the use of bacillus strains for promoting plant growth can provide a great option for increasing plant health and yields in greenhouse conditions. Although the generality of the idea of PGPR-host relationships is that bacterial strains are found to be more effective for promoting plant growth when they are applied on plants from which they were isolated, I will say that PGPR strains isolated from one crop can be used to increase the growth of a different crop plant. The strain might even display higher growth promotion efficacy on the non-host crop compared to

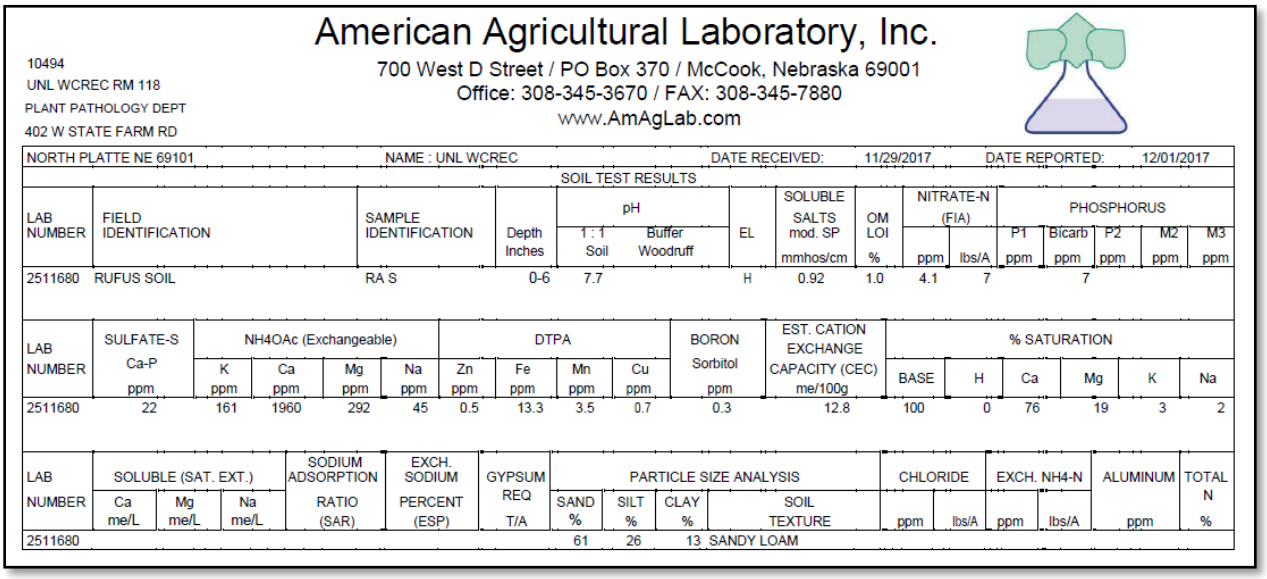
the host as found in this study. However, irrespective of which plant a potential PGPR strain was isolated, the strain should be tested across varieties of different crops to determine its effects on individual crop variety. Also, I will recommend that screening of potential PGPR strains is better done using direct greenhouse pot assays than to use *in vitro* screening procedures. The use of *in vitro* screening assays might lead to selecting a 'false' potential PGPR strain while rejecting a 'true' potential PGPR. Considering the results that some of the strains that were effective *in vitro* were inefficient *in vivo* and vice versa is a good point that supports this recommendation. Another point is that, it requires advanced effort, skills and technologies to conduct some of the *in vitro* assays whereas conducting pot experiments only requires comparatively lower demand of these items. Also, there is no need to search for the mechanisms of plant growth-promotion in a bacterial strain until such strain has been shown to increase plant growth in a soil system. That would prevent unnecessary waste of material resources, time and efforts that would have gone into conducting such unnecessary *in vitro* assays. Also, following this recommendation would provide opportunity for scientists and farmers in some environments such as in underdeveloped and developing countries where there is little or no access to expensive research facilities, technologies and technical know-how that would be needed for screening the bacterial strains *in vitro.* It would be easier in such environments to just apply cell suspensions of potential PGPR cultures directly on seeds and grow them in pots in glasshouse which does not necessarily require sterile or extreme controlled environment.

Further research studies requiring fields experiments are needed to further evaluate the bacterial strains' potentials and mechanisms for plant growth-promotion. Testing all the high efficient strains on corn, wheat, and soybean in field studies would be a great next step that would help to determine if the strains can increase the growth of the crops in natural environments. Besides the differences in environmental factors of greenhouse and fields, which may differently influence the growth stimulation effects of the strains, these crops require relatively up to three to four months to reach harvest, making it difficult to evaluate them for yield increase in a greenhouse. Hence, field studies would be helpful in testing if the strains would increase yields in the crops. Another test that can be conducted with the strains may involve testing the synergistic effects of two or more of the strains in soil systems. The cell suspensions of the strains would be applied onto seeds as mixtures (consortia) and observed for plant growth-promotion effects in field or greenhouse potted soils. Also, to support the idea of integrated nutrient management system and to reduce chemical fertilization (CF) pressure on agricultural soils, study can be conducted to compare different treatments such as "PGPR + reduced CF"; PGPR alone, Chemical fertilization, and non-treated control plant in greenhouse and field studies. Besides these studies, another study can be conducted to test specific strain for increasing the growth of mutant-plants defective in indole acetic acid or specific plant hormone absorption using potting mix. This would help to know if this *in vitro* trait can be expressed by the strains in soil environments. Also, a study can be conducted using calcareous soil— limited in plant available iron—to

further study the strains for ability to scavenge and mobilize iron for plants in iron-limited soil. The same kind of experiment can also be conducted to test for the expression of the phosphate solubilization ability *in vivo* using soil containing insoluble form of phosphate. I believe these suggestions provide great directions as to what can be done to realize the end goal of developing some of these strains into plant growth promoting products for improved crop production.

Appendix

Analysis of the soil mix used for the greenhouse experiment.



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SOIL TEST RESULTS

LAB NUMBER	FIELD IDENTIFICATION	SAMPLE IDENTIFICATION	Depth Inches	pH 1 : 1 Soil	pH Buffer Woodruff	EL	SOLUBLE SALTS mod. SP mmhos/cm	OM LOI %	NITRATE-N (FIA) ppm	NITRATE-N (FIA) lbs/A	PHOSPHORUS P1 ppm	PHOSPHORUS Bicarb ppm	PHOSPHORUS P2 ppm	PHOSPHORUS M2 ppm	PHOSPHORUS M3 ppm
2511680	RUFUS SOIL	RA S	0-6	7.7		H	0.92	1.0	4.1	7		7			

LAB NUMBER	SULFATE-S Ca-P ppm	NH4OAc (Exchangeable) K ppm	Ca ppm	Mg ppm	Na ppm	DTPA Zn ppm	Fe ppm	Mn ppm	Cu ppm	BORON Sorbitol ppm	EST. CATION EXCHANGE CAPACITY (CEC) me/100g	% SATURATION BASE	H	Ca	Mg	K	Na
2511680	22	161	1960	292	45	0.5	13.3	3.5	0.7	0.3	12.8	100	0	76	19	3	2

LAB NUMBER	SOLUBLE (SAT. EXT.) Ca me/L	Mg me/L	Na me/L	SODIUM ADSORPTION RATIO (SAR)	EXCH. SODIUM PERCENT (ESP)	GYPSUM REQ T/A	PARTICLE SIZE ANALYSIS SAND %	SILT %	CLAY %	SOIL TEXTURE	CHLORIDE ppm	CHLORIDE lbs/A	EXCH. NH4-N ppm	EXCH. NH4-N lbs/A	ALUMINUM ppm	TOTAL N %
2511680							61	26	13	SANDY LOAM						